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Safety of Malathion Dusting Powder for Louse Control *

WAYLAND J. HAYES, Jr, M.D., Ph.D.,[1] ARNOLD M. MATTSON, Ph.D.,[2]
J. GORDON SHORT, M.D.[3] & ROBERT F. WITTER, Ph.D.[4]

Because some strains of body lice are resistant to DDT and γ-BHC, there is need for other effective, safe chemicals to control them. Malathion is known to be effective at a concentration of 1%. To test its safety, the bodies and clothing of 39 men were dusted 5 times a week for 8-16 weeks with talcum powder containing 0, 1%, 5%, and 10% malathion. Complaints about odour and skin irritation were roughly proportional to dosage. No change in blood cholinesterase activity was found, except perhaps with 10% powder. Urinary excretion of malathion-derived material was proportional to dosage. No other changes attributable to malathion were observed and the compound is considered safe for control of head and body lice.

Human body lice are responsible for the transmission of epidemic typhus and louse-borne relapsing fever. Epidemics of typhus could be controlled to some extent before the advent of modern insecticides. However, such epidemics were stopped for the first time in history through the use of DDT dusting powder during the Second World War (Simmons & Upholt, 1951; Simmons, 1959). Lice resistant to DDT first became an important problem during the Korean War (Hurlbut et al., 1952), but powders containing 1% γ-BHC were successfully substituted for those containing 10% DDT. Powders containing DDT are still used successfully in some parts of the world, but γ-BHC is required in Korea and in many parts of Asia, Africa, and the Americas (Wright & Brown, 1957). However, because of the emergence of strains resistant to γ-BHC (Wright & Brown, 1957), there is a need for other chemicals of low toxicity to man which will kill lice. An organic phosphorus compound, malathion (S-(1,2-diethoxycarbonylethyl)-O, O-dimethyl dithiophosphate), has less dermal toxicity to animals than DDT in solution and is effective for louse control (Cole & Burden, 1956; Cole et al., 1958).

In view of the low mammalian toxicity of malathion and its effectiveness against lice, it seemed desirable to test the safety to man of anti-louse powder containing this insecticide. The safety of malathion was supported by very extensive use experience, some of which has been published (Bruaux, 1957), as well as extensive studies by Golz (1959) regarding the safety of human exposure to malathion aerosols. In addition, Cole et al. (1958) have carried out studies of anti-louse powders which were applied to limited areas of the body. When the design of the present experiments was complete, the very thorough study by the US Army Chemical Warfare Laboratories regarding the cutaneous application of 1.1% malathion to volunteers became known.[5] Later, Moeller & Rider (1959) reported giving 16 mg of malathion orally to 5 patients daily for 88 days without significant effect.

However, in none of the above studies was the effect of prolonged dermal application of powders containing high concentrations of malathion investigated, nor was measurement of the absorption of malathion attempted. Therefore, in the present studies volunteers were exposed for as long as

* From the Technical Development Laboratories, Technology Branch, Communicable Disease Center, Public Health Service, Bureau of State Services, US Department of Health, Education, and Welfare, Savannah, Ga., USA

[1] Chief, Toxicology Section, Technical Development Laboratories

[2] Chemist, Technical Development Laboratories

[3] Medical Officer, Technical Development Laboratories; now with the Department of Biological Chemistry, University of Utah, Salt Lake City, Utah, USA

[4] Biochemist, Technical Development Laboratories

[5] Gutentag, P. J. (1959) *CWL Report*, No. 2290 (unpublished report of the US Army Chemical Warfare Laboratories, Army Chemical Center, Maryland)

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16 weeks to 5% malathion, while others were exposed for 8 weeks to 10% malathion, which is 10 times the concentration proposed for use against lice. In addition, it was possible with the aid of a newly developed method (Mattson & Sedlak, 1960) to measure the excretion of ether-soluble metabolites of malathion in the urine. It was thus possible to obtain some idea of the actual amount of malathion absorbed from the skin.

Permission was obtained from the Bureau of Prisons, US Department of Justice, to carry out the experiment using volunteers in a Federal correctional institution. The study was explained in detail to those who indicated an interest in participating. So many were interested that many had to be eliminated by the drawing of lots. The men who tentatively volunteered were examined and were accepted if they proved to be qualified physically and in other ways. The fact that participation was at all times voluntary must be emphasized. Each man was free to leave the study at any time.

TABLE 1
DESIGN OF EXPERIMENT REGARDING SAFETY OF MALATHION

Season	Group No.	Malathion conc. (%)	No. of men	Case No.	Cumulative duration of participation (weeks)
Spring	1	0	10	1-10	8
	2	1	10	11-20	8
	3	5	10	21-30	8
Summer	4	0	4	5, 6, 9, 10	16
	5	1	6	15-20	16
	6-A	5	4	11,[a] 21-23	16
	6-B	5	6[b]	1, 2, 31-34	8
	7-A	5[c]-10[d]	4[b]	27-30	16
	7-B	10	6[b]	4, 35-39	8

[a] Case 11 received 1% powder during the spring; others received 5% powder throughout.
[b] Each of these groups included two Negroes (subjects 29, 30, 33, 34, 36, 37).
[c] Spring.
[d] Summer.

METHODS

The selected volunteers were asked to choose partners; then the pairs were arranged into groups. Three groups of 10 men each entered the study in February 1959 and four groups of 4-10 men entered (or continued in) the study in May of the same year. In the first, or spring, series the men were assigned to groups by dealing cards, each bearing the name of two partners. In the second, or summer, series those partners who had both participated during the spring were left together; new men and men who no longer had their original partners were paired arbitrarily. In the summer series, all of the pairs were placed in groups arbitrarily in order that some men would receive a substantial dosage of malathion for the first time and others who had already received such a dosage for just over 8 weeks would continue to receive the same or a higher dosage for an additional 8 weeks. The design of the experiment is summarized in Table 1. When the various data for the summer series were analysed, no difference could be detected between subgroup 6-A, who had been exposed during the spring, and subgroup 6-B, who were first exposed during the summer. The same was true of subgroups 7-A and 7-B. For that reason, no distinction is made between the subgroups in the remainder of this report.

The malathion was formulated in talc at concentrations of 0, 1%, and 5% during the spring (for groups 1-3, respectively) and 0, 1%, 5%, and 10% during the summer (for groups 4-7, respectively) For 5 days per week each volunteer received a paper bag bearing his name and containing 90 g of the appropriate formulation. Every week-day morning, after taking a shower bath and contributing a sample of blood, if that were required, each man proceeded to a room with 15 cots where the partners dusted one another, covering the entire body except the head, neck, and genitalia with powder. Such thorough dusting requires only 25-30 g of talc. All the remaining powder was sifted into the clothing. After dusting, each man washed his hands and forearms and then went about his ordinary duties until the following morning, except that urine samples were collected each night from six of the men in turn. The dusting was done under direct supervision during the first two weeks of the spring series and during the entire summer series.

At the beginning and end of his participation, each man was given a careful symptoms review (involving 195 printed questions), a general physical examination, and a variety of laboratory tests. Special attention was paid to the neurological

examination. Among other laboratory studies, the bromsulfalein (sulfobromophthalein sodium) retention was determined using a dosage of 5 mg/kg and an interval of 30 minutes between injection and sampling blood from the opposite arm. Because mild poisoning by organic phosphorus compounds can be detected earliest by depletion of blood cholinesterase activity, this activity was measured several days per week for the first few weeks of each series and at weekly intervals thereafter. In order to relate the exposure of the volunteers to their actual absorption of malathion, the malathion-derived material in the urine was measured.

Although one of us (J. G. S.) had received, without injury, a 30-hour application of 10% malathion powder in preparation for the study of volunteers, it was not known with certainty that the repeated application of malathion would not lead to a depletion of blood cholinesterase within a few days. It was necessary, therefore, to choose a procedure for measuring cholinesterase activity which one man, who had other duties as well, could carry out on 30 blood samples on the same day the samples were collected. For this purpose, the Nelson procedure [1] was selected. It is carried out on whole blood but, because acetyl β-methyl choline bromide is used as a substrate, the activity of red cell or "true" cholinesterase alone is measured. In order to check the results of the Nelson method and to determine any effect of malathion exposure on plasma cholinesterase, duplicate samples were shipped to Savannah, Ga., under refrigeration just before the first dose of malathion and again on the 17th, 64th, and 73rd days of dosage; the red cell and plasma enzymes were measured by the method of Michel (1949). For convenience, the final samples (113th and 115th days of dosage) were determined in Savannah by the Michel method only. Regardless of the analytical method, all samples were heparinized. The values obtained by the Nelson procedure were corrected for a change in pH of the buffer (0.2 Δ pH/hour) which resulted from the addition of the acetyl β-methyl choline to the buffer. This blank was determined with each set of analyses. Packed red cell volumes were determined at the beginning and end of each part of the experiment, using Wintrobe haematocrit tubes. However, the results by the Nelson procedure were not corrected to a standard cell volume because, in confirmation of previous results,[2] it was found that variation in packed red cell volume for a given individual was less than the variation in the level of red cell cholinesterase in the same person.

Malathion-derived material in urine was determined by the method of Mattson & Sedlak. Briefly, the method consists of determining the amount of organic phosphate extracted by ethyl ether from acidified urine. Normal urine contains no detectable amount of such ether-extractable compounds. The organic phosphates are oxidized with perchloric acid, and the orthophosphate liberated is measured colorimetrically by a molybdate method. Urine samples were collected while each volunteer was in the hospital overnight, which was approximately every sixth night for any one man. The sampling period (usually about 6 hours) was timed after initial voiding. The volume and duration of collection of each sample were recorded. The samples were preserved by 1 ml of 28% formaldehyde for each 500-ml bottle. Shipment required two days or less. At the laboratory, all samples were refrigerated pending analysis.

Malathion used for the study was of premium grade, kindly provided by the American Cyanamid Co. It was guaranteed to contain 95% actual malathion. The longest holding period at the laboratory prior to formulation was 60 days. In every instance, the malathion was formulated the week before it was used so that the maximum age of the preparation at the time of use was 12 days. There was no indication that the formulations underwent any change even though they were shipped and held in ordinary paper bags, folded, but not sealed at the top. In formulation, the amount of malathion necessary for treating a particular group for a week was carefully weighed and dissolved in enough ethyl ether to make a total volume of 1200 ml. No agent for masking odour was used. The ether solution was allowed to trickle slowly from a separating funnel into a weighed quantity of talc (4590 g for groups of 10 men) while the sample was constantly stirred by a large, commercial food mixer. The addition of solution required about 30 minutes. The ether evaporated chiefly during this period and completely during at least 10 minutes of additional mixing. It was necessary only to set up a fan to blow the fumes away. Lots of

[1] Nelson, S. R. (1953) *CW Scientific Report*, No. 1 (unpublished report from the Medical Investigation Branch, Dugway Proving Ground, Utah)

[2] Klonglan, E. D., Denny, G. H. & Morris, J. F. (1956) *Dugway Proving Ground Report*, No. 109 (unpublished report from the Medical Investigation Branch, Dugway Proving Ground, Utah)

TABLE 2

ANALYSIS FROM OFFICIAL FAHRENHEIT TEMPERATURE RECORDS DURING THE PERIOD OF DOSAGE, 4 MARCH THROUGH 25 JUNE 1959

	March	April	May	June
Maximum daily temp. range	59°-82°	63°-86°	74°-95°	77°-93°
Mean ± SD [a]	70° ± 6.3°	83° ± 5.7°	86° ± 5.1°	86° ± 4.7°
Minimum daily temp. range	32°-61°	39°-67°	55°-72°	67°-79°
Mean ± SD [a]	49° ± 7.8°	59° ± 7.1°	65° ± 4.8°	70° ± 2.2°
Average daily temp. range	50°-72°	51°-76°	69°-81°	74°-83°
Mean ± SD [a]	60° ± 6.2°	71° ± 5.6°	76° ± 3.1°	78° ± 2.3°

[a] SD = standard deviation.

90 g of the formulated powder were placed in small paper bags. Each bag was prominently labelled with a volunteer's name to prevent confusion.

The plan of the experiment called for prolonged treatment of the men with malathion and also for a comparison of the effect of exposure during cool and warm weather. A summary of the temperatures during the period of actual treatment is given in Table 2.

RESULTS

General clinical observations

As might be expected, the initial symptoms review revealed a number of complaints such as headache, intolerance of certain foods, allergy, and others. However, there was no indication from a personal interview of the men or from the physical or laboratory examinations that these complaints were the result of significant illness or were more prevalent in one group than another. There was little variation and no systematic change in these complaints during the course of the experiment with the exception of the appearance or increase of two complaints: burning of the skin and visible dermatitis. Even these complaints were minimal during the spring series. The odour of the more concentrated formulations was generally evident during the spring as well as the summer. Furthermore, the more concentrated formulations differed from plain talc in appearance, flowability, and "feel". These were unavoidable handicaps which tended to reduce the clinical value of having a control group. However, at worst, the groups were incompletely recognized by the participants. This was partly because the men had some difficulty determining the exact source of odour since the odour was most pronounced at the time of application of the powder and all the formulations were applied in the same room at the same time. Furthermore, the men were purposely misled before the experiment began by the statement that although malathion has an odour, an even more odorous material *might* be added to the less concentrated formulations. (This procedure was actually considered but it was never put into effect because it could have complicated the study.) It thus happened that each of two men (Cases 3, 10) who remained in the control group throughout the experiment complained of the odour of his formulation, while another (Case 9), also in the control group, stated that the formulation caused burning and was worse on tender skin. One of these same men (Case 3) thought that he was a little more sleepy when first exposed (to the plain talc). These results confirm what is known about the power of suggestion, and oblige one to accept the symptoms of those who were actually exposed to malathion with some reservation. In line with such reservation, it is a fact that the strongest complaints about the odour came from a man (Case 17) in the 1% group and two (Cases 21, 32) in the 5% group rather than from any in the 10% group. In spite of such reservation, it must be noted that while two of 10 men complained of odour while they were in the control group, six of 10 receiving 1% malathion and all of those getting 5% and 10% malathion made such a complaint. In a similar way, one of 10 receiving plain talc complained of burning, itching or some similar discomfort while four of 10 receiving 1% and seven of 10 and six of 10 getting 5% and 10% malathion, respectively, eventually had this complaint. Only two men considered the burning really uncomfortable, and they were in the 5% and 10% malathion groups, respectively.

Even those in the 5% group made very little complaint during the spring series. On the contrary, complaints were common at the end of the summer series. (However, one man (Case 19) who got 1% malathion in both series found the first worse than the second.) The change was probably based in part on a real difference related to temperature and may even have been caused mostly by tempera-

ture *per se* since the relatively few complaints from the controls came chiefly in the summer. It was frequently remarked that the burning was worse on tender skin and worse with sweating, but that it did not last long after application of the powder. Although free to stop at any time, each man continued in the series (or two series) for which he volunteered. One of them seemed to express the feeling of the group when he said he would not want to wear such powder to see his girl-friend but he wouldn't mind it in a situation where it was necessary and everyone used it.

There were three cases of rash during the experiment, all in men exposed to 5% or 10% malathion. One (Case 11) was an erythematous rash around the umbilicus; it appeared during malathion application and regressed only partially during continued exposure. A papular rash on the shoulders and upper arms of Case 4 during the spring, while he was getting no malathion, was replaced by an erythematous maculopapular rash in the presternal area during the summer, when he got 10% malathion. This volunteer apparently considered both rashes the same and attributed them to dosage. One man (Case 32), who was at first disqualified because of a severe acne on his shoulders and back, was finally admitted to the experiment. He thought that the acne became better during exposure (5% malathion), and this appeared to be true.

In addition to these cases, there were several involving dermatophytosis. A rumour developed that malathion was an effective cure for athlete's foot, and some put large amounts of powder into their socks. Evidence for the cure was clinical, not statistical.

Important physical and laboratory findings for volunteers before exposure and at the end of each series in which they participated were tabulated.[1] Although several abnormal values appeared in the ranges, they showed no tendency to correspond to dosage groups or to become progressively worse during exposure. The haemoglobin, which tended to be a little low at first, dropped slightly during the summer. This was possibly related to temperature and was certainly not related to dosage. The white blood cell count was high in several men before exposure, but tended to return to normal. This was associated with a reduction in upper respiratory infection.

Results of the bromsulfalein test remained normal (less than 10% retention) throughout.

Blood cholinesterase

The red cell cholinesterase values for each group are shown in Fig. 1 and Table 3.[2] A Student's *t*-test (as modified by Fisher, 1924) showed that red cell values determined on paired samples by the methods of Nelson and Michel, respectively, could not be distinguished. For that reason, both sorts of value are shown in the figure. The fluctuations in the level of cholinesterase, which are clearly evident in the figure, cause some difficulty in determining whether a minor change in red cell cholinesterase was produced by any dosage of malathion. The possibility of a major change is excluded by the similarity of the average values for the experimental groups to the average value for the control group on the same day and also by the fact that the lowest average value observed at any time for any group was 0.52 Δ pH/hour, a value not far from normal. In attempting to determine the statistical significance of the very small changes which did occur, it is best to compare separately the values obtained for the different groups during the next-to-last week of each series, when the exposure of the men was drawing to a close, and during the last week, when the exposure was complete. The results of this comparison by means of Student's *t*-test are shown in Table 4. It may be concluded from the low and inconsistent values in Table 4 that concentrations of 1% and 5% malathion produced no significant change in red cell cholinesterase while 10% malathion produced a depletion which approached, but did not reach, statistical significance. The results of the present experiment do not permit one to predict the minimal dosage necessary to produce a statistically significant depletion or the larger dosage necessary to produce a clinically significant depletion.

A statistical study of groups, such as that described above, will not fully reflect the presence of a small proportion of values basically different from the population as a whole. Only a few of the men exposed to malathion exhibited a variation in their individual red cell cholinesterase assay which was greater than that to be expected in a normal population. Klonglan et al.[3] concluded from a statistical

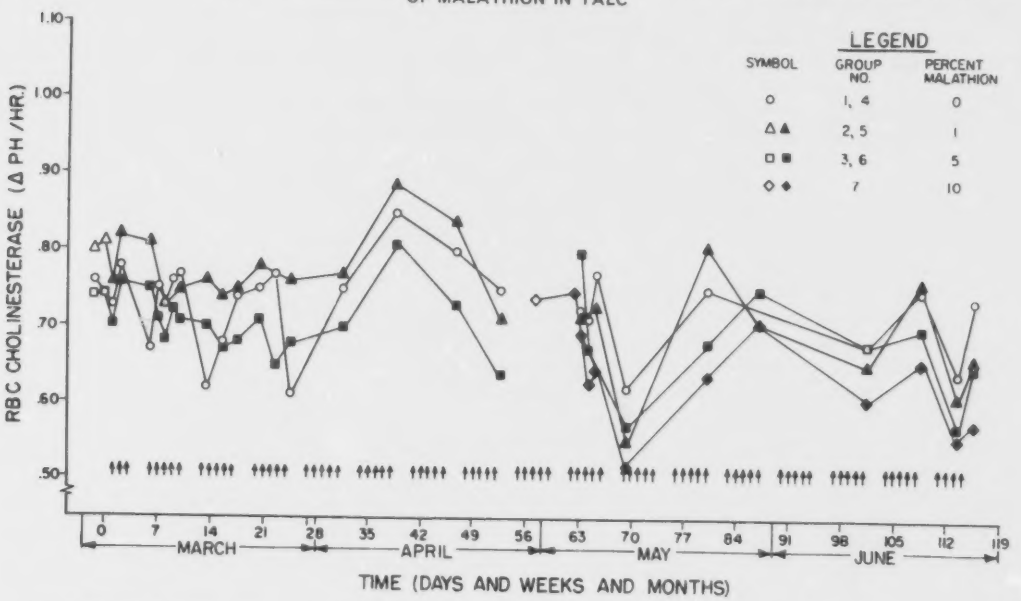
[1] Two tables have been deposited in the WHO Library and copies may be obtained on request.

[2] An extended form of this table giving the range, mean, and standard deviation of the values for each person concerned has been deposited in the WHO Library. Copies may be obtained on request.

[3] *Op. cit.*—see footnote, p. 503.

FIG. 1

AVERAGE RED BLOOD CELL CHOLINESTERASE OF GROUPS OF MEN EXPOSED TO DIFFERENT CONCENTRATIONS OF MALATHION IN TALC



Open points indicate no exposure; solid points indicate exposure; arrows indicate time of dosage

analysis of their results that the minimal true reduction in an individual cholinesterase level that can be detected after six or more determinations is 19%. Analysis of the data for individual volunteers showed that there were 15 instances in which minimal values for cholinesterase were less than 80% of the average for the same person. However, 10 of these lower assays were within 75% of the respective

TABLE 3

RED CELL CHOLINESTERASE LEVELS OF MEN EXPOSED TO MALATHION

Group No.	Malathion (%)	No. of determinations	Cholinesterase (Δ pH/hr)	
			Range	Mean ± SD [a]
1	0	99	0.54 - 0.99	0.75 ± 0.09
2	1	180	0.65 - 1.08	0.79 ± 0.10
3	5	207	0.42 - 1.13	0.71 ± 0.11
4	0	30	0.60 - 0.94	0.72 ± 0.08
5	1	40	0.51 - 0.88	0.69 ± 0.08
6	5	108	0.42 - 1.01	0.68 ± 0.11
7	10	130	0.40 - 0.87	0.64 ± 0.12

[a] SD = Standard deviation

TABLE 4

STATISTICAL SIGNIFICANCE OF DIFFERENCES IN RED CELL CHOLINESTERASE VALUES ASSOCIATED WITH DOSAGE OF MALATHION

Season	Comparison		Statistical level of difference (%)	
	Group No.	Concentration of malathion (%)	Penultimate week	Last week
Spring	1 *vs* 2	0 *vs* 1	< 60	< 20
	1 *vs* 3	0 *vs* 5	< 90	95
Summer	4 *vs* 5	0 *vs* 1	< 10	< 95
	4 *vs* 6	0 *vs* 5	< 60	< 95
	4 *vs* 7	0 *vs* 10	< 90	99

TABLE 5
EXCRETION OF MALATHION-DERIVED MATERIAL IN URINE OF MEN EXPOSED TO MALATHION

Group No.	Malathion (%)	Malathion equivalent (mg/hour)	
		Range	Mean ± SD [a]
1	0	None	None
2	1	0.03 - 1.17	0.27 ± 0.21
3	5	0.14 - 3.21	0.97 ± 0.64
4	0	None	None
5	1	0.02 - 0.93	0.30 ± 0.19
6	5	0.04 - 9.57	1.74 ± 1.65
7	10	0.09 - 5.80	1.99 ± 1.25

[a] SD = standard deviation

individual average. Of the five remaining low values, one was observed in each of groups 3, 4, and 7, respectively, and two in group 6. Furthermore, it should be pointed out that the low assays were observed on the 69th experimental day when, as is shown in Fig. 1, all groups tended to have minimal cholinesterase except for Case 25, whose lowest cholinesterase assay was noted on the first pre-exposure day. In addition, these minimal cholinesterase levels were followed by a rise back to a random distribution about the average for a given individual. Thus, although this drop was noted most in individuals exposed to malathion, the exact significance of the decrease is questionable.

Measurement of the plasma cholinesterase activity by the method of Michel showed that no significant change occurred in any person in the experiment. The lowest observed value, irrespective of group, was 0.50 Δ pH/hour (Case 38), a value within the range of normal (Rider et al., 1957).

Excretion of malathion-derived material

The excretion of malathion-derived material in the urine of volunteers is shown in Table 5.[1] Analyses of urine samples taken from men in the control group gave uniformly negligible values which probably reflect slight cross-contamination of the volunteers with one another's powder. These values are not shown in the table but are shown in Fig. 2. The highest observed concentration of ether-extractable phosphorus in any urine sample was 107 p.p.m. calculated as malathion. The highest observed rate of excretion was 9.57 mg/hour.

The results for groups which actually received malathion were characterized by considerable variation between individuals and a very striking variation between the separate daily results for certain men in a single series. The variation between men is indicated by the fact that the highest average excretion in each of five groups exceeded the lowest average excretion for the same group by a factor of 2.6, 3.3, 2.2, 8.7, and 3.1, respectively. The variation between men is also indicated by the fact that each one tended to remain consistent (either low or high in relation to his group) from one series to another, as shown in Table 6.

At the higher dosage levels, some, but not all, of the observed variation between the amount of excretion of men in the same group could be explained by their difference in size and, specifically, in body surface. Table 7 shows the coefficient of correlation between body surface and excretion of malathion-derived material for the groups under study. The coefficients for groups receiving 5% and 10% malathion ranged from 0.43 to 0.64. No explanation (such as uniformity of one or both of the variables involved, was found for the absence of correlation between surface area and excretion for the group receiving 1% malathion. It is, of course, true that with small statistical samples such as these, a single value may make a great difference in the coefficient.

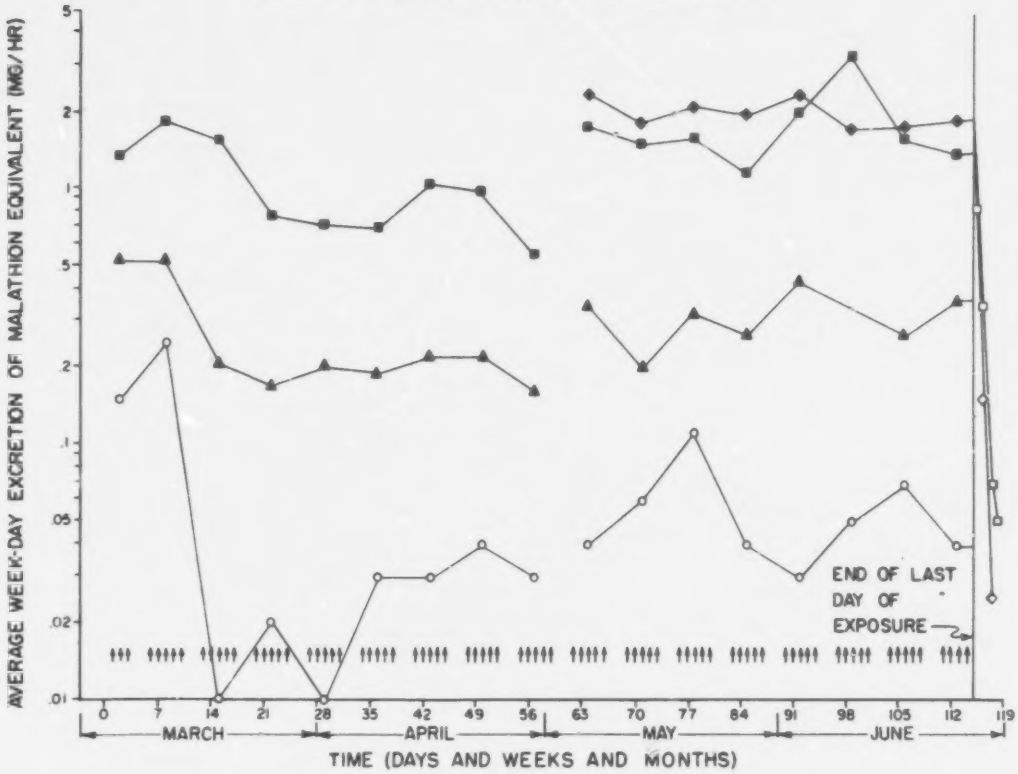
In contrast to the variation between individuals in the same group, no explanation was found for the variation between repeated observations on the same man. This latter variation may have been random in character. It was not related significantly to the chemical determination which gives repeatable results for a given sample. The great difference in the coefficient of variation (21%-144%) for repeated observations on individual men suggested that some men were inherently more variable than others in the excretion of malathion-derived material. However, as shown in Table 6, this was not generally true, because some men showed a marked increase and others a marked decrease in variability when their values for the spring and summer series were compared.

Other potential sources of variation in excretion would be duration of exposure or seasonal temperature. The average excretion of malathion equivalent

[1] An extended form of this table, giving the range, mean and standard deviation of the values for each person concerned, has been deposited in the WHO Library. Copies may be obtained on request.

FIG. 2

AVERAGE EXCRETION OF MALATHION-DERIVED MATERIAL IN THE URINE OF GROUPS OF MEN EXPOSED TO DIFFERENT CONCENTRATIONS OF MALATHION IN TALC



Symbols and conventions as in Fig. 1.

by the different groups as a function of time is shown in Fig. 2. It may be seen that excretion was relatively high early in the spring but decreased after 2-3 weeks; it increased promptly when the summer series began and continued at a high rate during the remainder of the experiment. These changes were unrelated to temperature or duration of exposure but clearly reflected the degree of supervision which was possible as the experiment progressed. Further indication of the tendency to greater excretion during the period of better supervision is shown in Table 6, which compares the spring and summer performance of six men who continued to receive 1% powder and three men who continued to receive 5% powder. The average excretion by the first group during the summer was 1.3 times their excretion during the spring. The corresponding factor for the second group was 1.1. Men who received 5% powder during the spring and 10% powder during the summer excreted 2.2 times as much malathion derivative while on the higher dosage. If one subtracts the variation between the two series, which can be attributed to supervision, the factor becomes exactly 2.0.

The difference between white and Negro men in the excretion of malathion-derived material was not statistically significant ($P=>0.7$). However, four of the six Negroes had relatively low values for their respective groups. It is possible that a statistical difference could be demonstrated if larger

TABLE 6

COMPARISON OF THE EXCRETION OF MALATHION-DERIVED MATERIAL IN THE URINE OF INDIVIDUAL MEN AS RELATED TO DOSAGE AND SEASON

Malathion concentration (%)		Case No.	Excretion calculated as malathion					
			Spring series			Summer series		
Spring	Summer		Range (mg/hour)	Mean ± SD [a] (mg/hour)	C [b]	Range (mg/hour)	Mean ± SD [a] (mg/hour)	C [b]
1	1	15	0.03 - 0.48	0.22 ± 0.15	68	0.05 - 0.38	0.20 ± 0.12	60
		16	0.03 - 0.64	0.18 ± 0.26	144	0.02 - 0.65	0.23 ± 0.22	96
		17	0.11 - 0.54	0.26 ± 0.17	65	0.13 - 0.78	0.41 ± 0.19	46
		18	0.04 - 0.80	0.27 ± 0.23	85	0.16 - 0.45	0.28 ± 0.095	37
		19	0.05 - 0.43	0.23 ± 0.11	48	0.21 - 0.93	0.44 ± 0.25	57
		20	0.08 - 0.46	0.19 ± 0.12	63	0.16 - 0.45	0.27 ± 0.12	44
		Group	0.03 - 0.80	0.23 ± 0.17	74	0.02 - 0.93	0.30 ± 0.19	63
5	5	21	0.26 - 1.19	0.50 ± 0.31	62	0.10 - 1.31	0.47 ± 0.39	83
		22	0.45 - 1.91	1.29 ± 0.58	45	0.10 - 5.88	1.86 ± 2.08	112
		23	0.24 - 1.55	0.69 ± 0.43	62	0.10 - 0.67	0.51 ± 0.31	61
		Group	0.24 - 1.91	0.84 ± 0.56	67	0.10 - 5.88	0.95 ± 1.35	142
5	10	27	0.87 - 1.71	1.18 ± 0.25	21	0.92 - 3.10	1.67 ± 0.67	40
		28	0.68 - 1.42	1.08 ± 0.25	23	0.81 - 5.20	3.13 ± 1.37	44
		29	0.59 - 2.30	1.22 ± 0.62	51	1.74 - 3.43	2.81 ± 0.58	21
		30	0.16 - 0.59	0.39 ± 0.16	41	0.12 - 2.23	1.06 ± 0.77	73
		Group	0.16 - 2.30	0.97 ± 0.49	51	0.12 - 5.20	2.15 ± 1.22	57

[a] SD = standard deviation.
[b] C = coefficient of variation.

TABLE 7

COEFFICIENT OF CORRELATION BETWEEN BODY SURFACE AREA AND EXCRETION OF MALATHION-DERIVED MATERIAL

Group	Concentration of malathion (%)	Coefficient of correlation
2	1	− 0.03
3	5	+ 0.57
5	1	+ 0.03
6	5	+ 0.64
7	10	+ 0.43

subgroups were used. In fact, counting both spring and summer cycles, each of the four contributed at least one value that was smaller than that for any white man in the same group.

During the spring series, the average excretion of malathion-derived material by men receiving 5% malathion powder was 3.6 times greater than the average excretion by men receiving 1% powder. The corresponding values during the summer series differed by a factor of 5.8. The men who received 10% malathion during the summer excreted malathion-derived material at a rate 6.6 times as great as that of men receiving 1% powder during the same season. It has already been mentioned that men who got 5% malathion in the spring and 10% in the summer excreted twice as much malathion-derived material during the summer. In view of the variation mentioned above, it is concluded that these results indicate that the excretion of malathion-derived material is proportional to dosage at the levels studied. There was no evidence that the excretion (or absorption) of malathion reached a plateau as the dosage level was increased. This

does not exclude the possibility that a plateau of absorption might be reached if some concentration of malathion higher than 10% were applied to the skin.

It was not practical in the present experiment to collect serial samples of urine to cover an entire 24-hour period. As already noted, there was no trend toward increasing excretion after the first day of exposure. In preliminary experiments on one of us (J. G. S.) with 1%, 5% and 10% powder, serial urine samples were collected. In every instance, excretion of malathion-derived material reached an irregular plateau after 6-8 hours. After bathing, excretion decreased rapidly so that only traces of malathion-derived material were detectable in the urine 8 hours later. Loss of malathion from the body may be slightly slower following repeated daily exposure than after a single application; however, complete excretion of this compound requires only a few days at most. In the present experiment, the average excretion of malathion-derived material was 18% (as compared to the week-day rate) for urine collected on Saturday night (one day after last dose of malathion) and 7% for urine collected on Sunday night (two days after last dose of malathion). At the end of the experiment, excretion decreased at about the same rate as on week-ends and reached levels indistinguishable from the blank by the third day.

TABLE 8

PERCENTAGE OF MALATHION RECOVERED FROM URINE [a]

Group No.	Malathion (%)	Lowest		Highest		Mean for group
		Single value	Mean for man	Single value	Mean for man	
1	0	—	—	—	—	—
2	1	0.28	1.8	11.0	4.3	2.5
3	5	0.26	0.7	6.1	2.4	1.8
4	0	—	—	—	—	—
5	1	0.19	1.9	8.8	4.2	2.8
6	5	0.08	0.9	18.0	7.7	3.3
7	10	0.09	0.9	5.5	3.0	1.9

[a] The calculation is made on the basis that 28 g of formulation were really available for absorption. The percentages given in the table must be multiplied by 0.278 in order to put them on the basis of the 90 g of formulation actually issued to the men.

DISCUSSION

There is some interest in attempting to measure the percentage of the total dosage of malathion which was recovered in the urine. Each man was issued 90 g of powder each day. However, it is quite impossible to apply 90 g of talcum powder to the skin. When one reaches 25 g or 30 g, the powder begins to fall off as fast as it is applied. There is, therefore, some reason to use 28 g (a high average within the observed range) as the greatest weight of powder available for absorption at any one time.

Daily excretion of malathion during the week-end averaged 0.28 times the excretion on a week-day. This additional recovery of malathion equivalent has been taken into account in calculating the proportion of the dose recovered: that is, week-day values or averages have been multiplied by $(5+(2\times0.28))/5$ or 1.1. Table 8 shows the percentage of malathion equivalent actually recovered from the urine of different groups of men. It may be seen that the average values for different groups ranged from 1.8% to 3.3% of the malathion available for dermal absorption. These values may be compared with 23% determined in man for excretion and therefore absorption of malathion taken orally in a single dose at the rate of 0.85 mg/kg (Mattson & Sedlak). The fact that nearly a quarter of the oral dose was recovered from the urine indicates that the observed values do not differ from true values by a factor greater than 4. Studies by the same authors using radioactive malathion indicate that, at least in rats, the method detects an average of 66% of the total malathion-derived material present in the urine. Although excretion in man may be different from that in the rat, it appears likely that only a portion of the malathion-derived material in human urine is detected by the available chemical method. Thus, the upper limit of true average absorption of malathion applied to the skin as a powder is probably slightly less than 10%, and the lower limit is about 4%. However, a few people probably reach much higher values, as indicated by an observed (uncorrected) average value of 7.7% in Case 32, whose highest single observed value was 18.0%.

The average values in Table 6 compare favourably with corresponding values for preliminary tests

carried out on one of us (J.G.S.). This is evidence that the volunteers received their intended exposure.

This agreement of results is also of interest in discussing the possibility of oral or respiratory intake of malathion incidental to the intentional dermal exposure. The head was undusted and the hands and forearms were washed promptly after dusting as a precaution against the contamination of food, water, and tobacco or direct contamination of the nose or mouth. Oral or respiratory intake would have been difficult to measure under the conditions of the experiment and no attempt was made to do so. There was no objective reason to think that these routes of entry were significant either in the preliminary tests or in the final study.

CONCLUSION

Malathion is considered safe for the control of human head or body lice, especially since infrequent applications of small amounts of 1% powder are effective. The safety of malathion for other purposes, including those that may be developed in the future, is related to absorption of the compound, and this may be measured indirectly in terms of malathion-derived material in the urine.

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RÉSUMÉ

Certaines souches de poux sont devenues résistantes au DDT et au HCH, d'autres le sont au lindane. Pour remplacer ces insecticides dans la lutte contre les maladies transmises par les poux, on a proposé le malathion, peu toxique pour les mammifères. Les auteurs rendent compte d'études faites en vue de déterminer la toxicité du malathion pour l'homme à la suite d'applications cutanées prolongées de poudres à fortes concentrations, et de mesurer le malathion absorbé.

La surface du corps et les vêtements de 39 hommes (répartis en groupes de 4 à 10 sujets) ont été traités cinq fois par semaine pendant 8-16 semaines avec une poudre de talc contenant 0%, 1%, 5% et 10% de malathion. La poudre a été laissée en place 23 heures par jour, mais elle a été enlevée durant les jours de fin de semaine. Les volontaires ont été examinés avec soin avant le début du traitement ainsi qu'à la fin des essais, effectués les uns au printemps, les autres en été. Au cours de ces deux saisons, le teneur sanguine en cholinestérase et le taux de métabolites du malathion dans l'urine ont été déterminés à maintes reprises.

La plupart des sujets traités ont remarqué l'odeur du produit et ont éprouvé une légère sensation de brûlure cutanée pendant quelques heures après l'application de la poudre surtout lorsqu'ils transpiraient. Les doléances concernant l'odeur et l'irritation cutanée étaient sensiblement proportionnelles aux concentrations employées, bien que le talc, appliqué seul, ait aussi provoqué des plaintes. Deux cas d'éruption bénigne ont été observés qui pouvaient être dus au malathion, mais on a également constaté l'amélioration d'une éruption préexistante, pendant l'exposition du sujet à une poudre de 5% de malathion. Malgré ces légers inconvénients, aucun des sujets traités ne se serait opposé à l'application de la poudre la plus concentrée, si elle avait été nécessaire à sauvegarder sa santé.

Les résultats des examens physiques et des analyses courantes n'ont revélé aucune anomalie. La fonction hépatique, évaluée par l'épreuve de rétention de la bromosulfo-phtaléine est restée normale.

Aucune modification importante de la cholinestérase dans le plasma n'a été observée. La poudre à 1% et 5% de malathion n'a pas modifié sensiblement le taux de cholinestérase des globules rouges, mais à la concentration de 10% elle a provoqué une déplétion atteignant un taux presque statistiquement significatif.

L'élimination des métabolites du malathion dans l'urine (par conséquent, l'absorption du malathion) a

été proportionnelle aux concentrations employées, mais a varié selon les sujets et, pour certains d'entre eux, d'un jour à l'autre. En moyenne, un peu plus de 2% de la quantité de malathion contenue dans la poudre ont été retrouvés dans l'urine, mais dans un cas, on a observé un taux d'absorption de 18%, correspondant à l'absorption de 9,7 mg de malathion par heure. Le malathion est rapidement excrété; il n'est plus décelable dans l'urine trois jours après la fin du traitement.

Le malathion utilisé dans la lutte contre le pou de tête et le pou de corps est considéré comme inoffensif pour l'homme, étant donné l'efficacité de la poudre à 1%, même en applications espacées de faibles quantités. La nocivité du malathion employé à d'autres fins dépend des quantités absorbées, lesquelles sont mesurables indirectement par dosage des métabolites du malathion éliminés dans l'urine.

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The Toxicology of Chemosterilants

WAYLAND J. HAYES Jr [1]

Sterilization of males can in certain circumstances be more efficient than killing as a method for control of insects and perhaps other pests. A number of chemicals (chemosterilants) show promise of producing sexual sterility in insects without some of the practical limitations of radiation. The most important compounds are alkylating agents. These have little immediate pharmacological action, but are notable for their selective action against haematopoietic and some other proliferating tissues. A number of alkylating agents have been shown to be mutagens in insects, bacteria, fungi, and higher plants; carcinogens in mammals; and teratogens in insects, birds, and mammals. Some produce sexual sterility, possibly in mammals as well as in insects, at doses too low to produce the other effects. Some have an established reputation as drugs for palliative treatment of leukaemia and other neoplasms.

The development of insect sterilization as a vector control technique has been handicapped in part by lack of scientific information on the acute and long-term hazards that might be associated with the use of chemosterilants. In this paper the author brings together the available knowledge on the toxicology of the alkylating agents.

INTRODUCTION

It is possible to show by mathematical models that sterilization of males is ultimately more efficient than killing as a method of controlling insects and perhaps other pests (Knipling, 1955, 1959). The reason is that, although both dead and sterile males fail to reproduce, some portion of the sterile ones will effectively prevent the reproduction of females that were not directly affected by the sterilizing agent. The degree of advantage will depend on many factors, including the reproductive physiology and ecology of the insect (Knipling, 1955). The housefly is especially suited to such control because both sexes frequently travel considerable distances before mating [2] and the sperm from the first mating, stored in the seminal receptacles, are used for all the remaining life of the female so that subsequent matings are ineffectual. Obviously, not every species is adapted to this kind of control. The suitability of the technique for the control of disease vectors has been discussed by Smith (1963).

The sterile-male technique requires that the treated male compete with other males and that the sterile sperm function normally enough to prevent subsequent effective mating and yet not normally enough to permit reproduction. No matter what agent is used, the dosage must be adjusted to cause just the right degree of injury. However, this is possible and with at least one compound (apholate) sterile male houseflies that were perhaps more competitive than normal ones have been produced (LaBrecque et al., 1962).

The concept of insect control or eradication through the use of sexually sterile males is credited to E. F. Knipling by Bushland & Hopkins (1951), who reported the first laboratory study of the method. The practicability of the technique was demonstrated in a series of brilliant experiments with the screw-worm fly (*Callitroga hominivorax*) sterilized by radiation. The species was first eradicated from the island of Curaçao (Baumhover et al., 1955) and later from Florida and the south-eastern United States (Knipling, 1960) with the result that farmers in the area are now free of this fly that formerly caused $20 million worth of damage to cattle each year. To achieve this eradication, screw-worm flies were grown under factory-like conditions; the pupae were irradiated from a cobalt source, and 3250 million adults which emerged were released

[1] Chief, Toxicology Section, Technology Branch, Communicable Disease Center, Public Health Service, US Department of Health, Education, and Welfare, Atlanta, Ga., USA.

[2] The males begin active mating on the second day and the females on the third day after emergence.

systematically over approximately 85 000 square miles (220 000 km²) during a period of 17 months (Lindquist, 1961).

An effort is now being made to broaden the method so that sterilization can be produced by a chemical rather than by radiation. A chemical used in this way is called a chemosterilant. Even if used in a " sterile-male factory ", the chemical approach might be cheaper and would certainly permit greater mobility of the " factory ". The insects could be reared near the point of use, reducing the stress of shipment and ensuring full vigour.

The degree of regulation that can be imposed on a control agent decreases as the degree of dispersal of that agent increases—i.e., from laboratory or " factory ", to baits or lures, to area treatment. Depending on whether safety can be ensured, the chemical approach might extend the ways of using the sterile-male technique to baits or lures and perhaps even to area treatment, which are completely impracticable in connection with gamma radiation. The advantages of having the insects rear themselves in nature rather than having to set up a factory for the purpose are obvious. Quite aside from expense, some species such as the tsetse fly are technically difficult to rear.

Even though the use of chemosterilants will necessarily be limited by the physiology of different insect species and by the toxicology of different chemicals, these agents promise to open up a new era in insect control (Knipling, 1959; Lindquist, 1961). What they will mean in terms of toxicology will depend on the compounds ultimately selected for use. The chemicals that have already shown significant value in laboratory tests fall into three classes: alkylating agents, antimetabolites, and miscellaneous compounds including some alkaloids (Borkovec, 1962, 1964). Eleven of them are shown in Table 1. Table 2 gives the chemical identity of other compounds referred to in the text by common name. An extensive list of promising compounds and a review of the entomological tests used in their evaluation have been presented by Smith et al. (1964).

The sterile-male principle is based on a single end-result and not on any one mode of action. However, the compounds that have received greatest attention are alkylating agents, and this brief review is confined largely to the toxicology of this class of compounds. Alkylating agents that are biologically active may be classified as sulfur mustards, nitrogen mustards, epoxides, etheleneimines, methanesulfonates, β-lactones, and possibly diazo compounds (Ross, 1958). Promising chemosterilants have been found among the phosphorus ethyleneimines and methanesulfonates. Possibilities may lie in other groups, notably the sulfur ethyleneimines, that have been little explored.

HISTORY OF THE ALKYLATING AGENTS

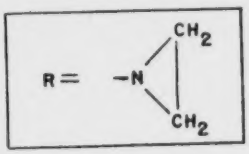
Significant interest in the chemical alkylating agents began with the application of mustard gas (bis(2-chloroethyl)sulfide) as an irritant and vesicant in the First World War. Although the systemic toxicity of mustard gas was observed during that war, little emphasis was placed on it by the majority of investigators. The advent of the Second World War, the inclusion of nitrogen mustard and its analogues among potential war gases, and the " Bari disaster " led to a careful reappraisal of the systemic toxicity of the group.

As a result of aerial bombardment on 2 December 1943, mustard gas from a cargo ship was mixed with the oil-slick coating the surface of the harbour of Bari, Italy. Many casualties, who were able to keep afloat in the water before rescue, were later kept wrapped in blankets until medical treatment was possible. The conditions favoured dermal absorption. Early casualties showed a shock-like syndrome that did not respond to treatment. A second peak of mortality associated with extreme leukopenia occurred eight to nine days after exposure. A survey left little doubt that the unique clinical findings represented systemic poisoning by mustard gas (Alexander, 1947).

Information gathered during the Second World War on the chemistry, biochemistry, pharmacology, and pathology of the alkylating agents led to the proposal that some of them be used in the treatment of cancer. Their success as palliative therapeutic agents led, in turn, to more detailed studies. Investigations of the mutagenic action of alkylating agents apparently was the independent result of academic exploration of external factors that influence heredity. Study of the carcinogenic action of alkylating agents may have followed from theoretical considerations about the relationship between mutagenic and carcinogenic action. The compounds were at one time considered in the search for antifertility substances for man and are now being studied actively as chemosterilants for insects.

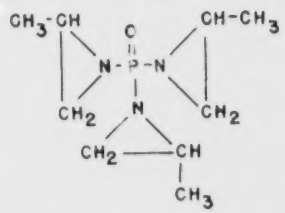
Some alkylating agents—for example, Dibenamine hydrochloride and phenoxybenzamine—have

TABLE 1
COMPOUNDS PROMISING AS CHEMOSTERILANTS



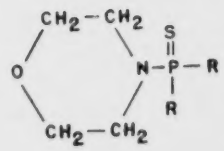
ALKYLATING AGENTS

1. **Metepa:** tris[1-(2-methylaziridinyl)] phosphine oxide (MAPO; methaphoxide; USDA ENT-50,003)

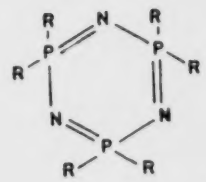


2. **Tepa:** tris(1-aziridinyl) phosphine oxide; triethylene phosphoramide (TEPA; TEF, aphoxide, SK-3,818; NSC-9,717; USDA ENT-24,915)

3. **Morzid:** bis(1-aziridinyl) morpholino phosphine sulfide; *N*-3-oxapentamethylene-*N'*,*N''*-diethylene thiophosphoramide (OPSPA; MSPA; NSC-10,429; USDA ENT-25,301)



4. **Apholate:** trimeric bisethyleneimido phosphonitrile; 2,2,4,4,6,6-hexahydro-2,2,4,4,6,6-hexakis(1-aziridinyl)-1,3,5,2,4,6-triazatriphosphorine (SQ 8388; Olin No. 2174; NSC-26,812; USDA ENT-26,316)



5. **Tretamine:** triethylene melamine; 2,4,6-tris(1-aziridinyl)-*s*-triazine; 2,4,6-triethyleneimino-*s*-triazine (TEM; TET; R-246; SK-1,133; M-9,500; Persitol Ho 1/193; triamelin; NSC-9,706; USDA ENT-25,296)

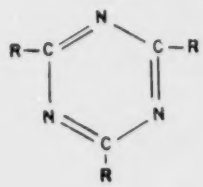
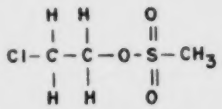


TABLE 1 (*continued*)

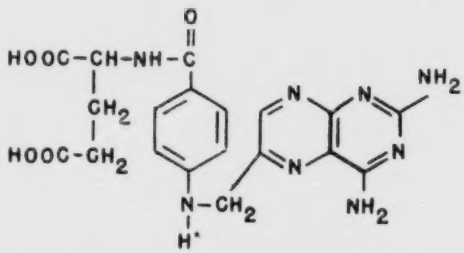
ALKYLATING AGENTS (*continued*)

6. **CB 1506:** 2-chloroethyl methanesulfonate
(NSC-18,016; USDA ENT-26,395)



ANTIMETABOLITES

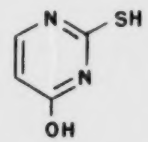
7. **Aminopterin:** 4-aminofolic acid; 4-aminopteroylglutamic acid; *N*-{*p*-[(2,4-diamino-6-pteridinyl)methyl]amino}benzoyl)-glutamic acid
(APGA; NSC-739; USDA ENT-26,079)



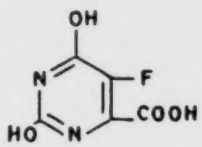
8 **Methotrexate:** 4-aminomethylpteroylglutamic acid; *N*-{*p*-[(2,4-diamino-6-pteridinyl)methyl)methylamino}-benzoyl}-glutamic acid
(amethopterin; A-methopterin; MTX; NSC-740; USDA ENT-25,299)

As above, but -CH_3 substituted for -H*.

9. **2-Thiouracil:** 2-mercapto-4-hydroxypyrimidine; 2-mercapto-4-pyrimidone
(USDA ENT-25,474)



10. **Fluoroorotic acid:** 5-fluoro-orotic acid
(RO 2-9945; NSC-31,712; USDA ENT-26,398)



MISCELLANEOUS

11. **Thiourea:** thiocarbamide
(USDA ENT-3,582)

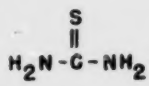


TABLE 2
CHEMICAL IDENTITY OF COMPOUNDS MENTIONED IN THE TEXT

Compound	Chemical identity
Dibenamine hydrochloride [a]	*N*-(2-chloroethyl)dibenzylamine hydrochloride
Phenoxybenzamine [b]	*N*-(2-chloroethyl)-*N*-(1-methyl-2-phenoxyethyl)benzylamine
Melphalan [b]	*p*-(*N*,*N*-di-2-chloroethyl)amino-L-phenylalanine
Busulfan [b]	1,4-dimethanesulfonoxybutane
Thiotepa [b]	triethylene thiophosphoramide

[a] A trade name.
[b] Proposed International Non-proprietary Name.

well-established use as adrenergic blocking agents (Nickerson, 1949). Their prolonged effects have been attributed, at least in part, to irreversible alkylation of adrenergic receptor sites.

At least on an experimental basis, vaccines prepared by treating Eastern equine encephalitis, fixed rabies, and hog cholera viruses with sulfur mustard successfully immunized test animals against challenging doses of the respective viruses (Tembroeck & Herriott, 1946). The use of methyl-bis(2-chloroethyl)amine is effective for the sterilization of stored blood or plasma but has certain theoretical disadvantages (Philips, 1950). Other uses have been considered in relation to the effects of alkylating agents on immune reactions (Philips, 1950).

TOXICOLOGY OF ALKYLATING AGENTS

Alkylating agents are notable for their delayed effect accompanied by a selective action against some proliferating tissues: haematopoietic cells of the bone marrow and lymphoid tissues, the intestinal mucosa, germ cells, embryos, and tumours. There are notable exceptions. For example, the earliest studies by Ehrlich (1910) and others (Gabriel & von Hirsch, 1896) revealed that the most striking pathology produced by ethyleneimine in the rat is complete necrosis of the renal papilla, a tissue that is not notably proliferative. The marked susceptibility of some embryonic cells is apparently not related to their mitotic activity (Murphy et al., 1958).

The following paragraphs describe the few acute and the many delayed effects.

Acute effects

Some of the sulfur mustards and nitrogen mustards have cholinergic, paralytic, and central-nervous-system stimulatory effects. The methanesulfonates can cause convulsions, and supralethal doses of tretamine are convulsant and paralytic (Philips, 1950). These actions may be the cause of early death if doses well above the LD_{50} are given to animals, and the actions may give rise to side-effects following therapeutic doses for man. Some of the mustards may produce the same clinical effect twice in succession following a single dose as the result of separate pharmacological actions (Philips, 1950).

Effects on haematopoiesis

Following a dose in the range of the minimal lethal dose, cessation of mitosis and disintegration of all but the most primitive elements appear in the lymphoid tissues and bone marrow within six to eight hours following mustards (Philips, 1950) or within a few days following the more slowly acting ethyleneimines (Sternberg et al., 1958). Changes in the circulating blood parallel those in the haematopoietic system. The lymphocytes tend to disappear first and recover last; the granulocytes and thrombocytes show an intermediate reaction; and anaemia, if it occurs, is delayed in appearance and only moderate in degree. Haematological recovery after toxic doses of radiation appears to be less rapid than that following comparable intoxication with mustards (Philips, 1950).

Effects on intestinal epithelium and other organs

Usually less striking than the blood changes are changes in the intestinal mucosa. Following mustards, atrophy and erosions may occur. Ethyleneimines produce similar but less prominent changes (Sternberg et al., 1958).

In addition to the destructive changes, hypertrophy of the adrenal may occur (Philips, 1950).

Delayed lethal syndrome

It might be supposed that the severe suppression of haematopoiesis and the anorexia, diarrhoea, and profuse vomiting that may accompany intestinal changes would be adequate to explain death that characteristically occurs three to seven days after a single dose in the LD_{50} range. However, there is no overwhelming sepsis and the fluid changes do not approach in magnitude the range associated with fatal shock following burns, trauma, or haemorrhage. By occluding a portion of the circulatory system for

only a few minutes, it is possible to protect the corresponding portion of the haematopoietic system or intestine from injury, and yet animals treated in this way go on to die after the same interval as animals given the same dose and no protection. However, bacteria-free mice are more resistant to the delayed lethal effects of nitrogen mustard than normal mice. This finding supports the hypothesis that intestinal bacteria play an important role in the systemic toxicity of such compounds, but the relative immunity of germ-free mice can be overcome by increased dosage (White & Claflin, 1963). It is true that analogues that do not derange proliferative tissues usually do not cause the delayed lethal syndrome (Philips, 1950). However, one analogue of sulfur mustard (β-chloroethyl-β-(bis(β-hydroxyethyl)sulfonium)ethyl sulfide chloride) causes delayed deaths with no involvement of the bone marrow (Anslow et al., 1948). As shown in the review by Philips (1950), no completely satisfactory explanation for the characteristic mode of death has been found.

The manner of death following repeated doses of alkylating agents resembles the delayed lethal syndrome following a single dose except that gastrointestinal signs and pathology are less prominent.

Mutagenesis

Modern concepts of mutation and the whole array of mutagenic agents have been reviewed by Zamenhof (1963), while the relation of chemical structure to mutagenic action has been reviewed by Röhrborn (1960). In addition to the alkylating agents, typical mutagens include radiation, heat, nitrous acid, hydroxylamine, various base analogues (e.g., substituted uracils), deuterium, formaldehyde, and even ferrous ion (Zamenhof, 1963). All types of genetic effects known from other sources, including X-ray and ultraviolet radiation, can be produced by chemicals.

Although the monofunctional alkylating agents are generally effective as antitumour agents only at concentrations 50-100 times those of polyfunctional compounds (Auerbach, 1958; Roberts, 1958), certain monofunctional mustards are even more effective genetically than the corresponding bifunctional ones (Auerbach, 1958). However, monofunctional compounds may be less efficient than difunctional ones of the same chemical series in inducing small deficiencies and deletions (Fahmy & Fahmy, 1959b). Although mutagenic action may affect proliferating cells, mustard gas is very effective in producing genetic change in mature sperm stored in the seminal receptacles of female *Drosophila*. On casual examination, the similarities between the genetic effects of X-rays and mustard gas are more striking than the differences. However, the more detailed the study, the more frequent and significant the differences appear (Auerbach, 1958; Fahmy & Fahmy, 1958). It has been shown that of two genes in the same nucleus, one responds preferentially to one mutagen, the second to another (Auerbach, 1958; Fahmy & Fahmy, 1958) and this is true even at a subgenic level (Fahmy and Fahmy, 1959a). Alkylating agents have produced visible mutations that were new in the sense of being different from those predominantly encountered in radiation experiments. The results indicate that some of the visible loci responsive to alkylating agents are less mutable or possibly even refractile to radiation (Fahmy & Fahmy, 1958). Treatment of mature sperm of *Drosophila* with either monofunctional or polyfunctional alkylating agents produces a higher proportion of mosaic mutations and a lower proportion of whole-body mutations than are produced by radiation under anoxic conditions (Oster, 1963).

Using a bacterial system to test mutagens, tepa, morzid and tretamine were found active (Szybalski, 1958). Successful studies have been carried out in higher plants as well as in bacteria (Philips, 1950; Ross, 1962), but chiefly in the fruit fly (*Drosophila melanogaster*) and in the slime mould (*Neurospora*) (Auerbach, 1958). In some studies, chemicals under test may be injected into the flies around the gonads (Fahmy & Fahmy, 1958) or used to soak the mould. Mustard gas, tretamine and CB 1506 are mutagenic in *Drosophila* (Auerbach & Robson, 1947; Fahmy & Fahmy, 1958). CB 1506 produces an unusually high proportion of visible mutations (Fahmy & Fahmy, 1956). Some of the surviving mutants have been sterile but others have transmitted morphological abnormalities to the F_2 generation (Auerbach & Robson, 1947).

Auerbach & Falconer (1949) speculated that a mutation in the progeny of mice treated with methyl-bis(2-chloroethyl)amine was caused by the treatment, but the senior author failed to confirm the important finding in a later review (Auerbach, 1958). Cattanach (1957, 1959b) reported that some of the F_1 progeny of male mice treated with tretamine showed hereditary semisterility and that this trait was associated with cytologically demonstrable chromosomal translocations. The semisterility was associated with survival of the sperm but death of the

zygote or embryo. He speculated that incomplete spermatogenesis and complete sterility in other F_1 offspring of treated male mice were caused by a dominant mutation. The injection of tretamine before mating also reduced the fertility of female mice, but a larger dose was necessary than in males. Cattanach (1959a) considered that the mechanism of action was the same in females as in males although direct evidence was not available. Only one F_1 progeny of treated mice was morphologically abnormal and it did not breed (Cattanach, 1959b). Apparently no transmission of a chemically induced morphological change in mammals has been reported.

Teratogenesis

Superficially similar in final result to mutagenic effects are the teratogenic effects observed in insects (Bodenstein & Abdel-Malek, 1949), mammals and birds (Murphy et al., 1958). At least one candidate chemosterilant, tretamine, and several related materials have been studied in this regard. For different compounds doses of 0.40 to 0.57 of the maternal LD_{50} in rats caused resorption of half of the litters (litter LD_{50}). Dosages less than about one-third of the litter LD_{50} produced no observable damage to the foetuses, while higher dosages produced more and more severe effects. For different compounds, dosages ranging from 1.3 to 2.0 times the litter LD_{50} were fatal to 100% of litters. Some antimetabolites, including aminopterin, were far more toxic to the foetuses. The litter LD_{50} for aminopterin was only 0.167 times the maternal LD_{50}, and this factor for 6-diazo-5-oxy-L-norleucine was 0.0019 (Murphy et al., 1958). There were some differences in the kind of injuries shown by rats surviving to term after treatment by different compounds, and few, if any, corresponded to the injuries produced by X-rays. More severe and consistent teratogenic effects were produced by X-rays.

It was possible to destroy the viability of sarcoma 180 by giving any of several alkylating agents at one-fifth the LD_{50} dose to 12-day chick embryos without great injury to the embryo. No such selective action was seen with X-rays, which damaged the tumour severely only at doses that killed the embryo (Murphy et al., 1958).

Lutwak-Mann & Hay (1962) proposed a rapid screening test for compounds that affect the early embryo when administered to the mother. Thiotepa gave positive results. Sokal & Lessmann (1960) reviewed the effects of cancer chemotherapeutic agents, including alkylating agents, on the human foetus. Busulfan and 6-mercaptopurine administered throughout pregnancy produced multiple severe abnormalities even though neither drug alone produced major foetal damage. There were no reports of important foetal damage in cases in which chemotherapeutic drugs were given only after the first trimester of pregnancy, and in fact these drugs have proved far less toxic than would be anticipated from animal experiments.

Carcinogenesis

Because many of the effects of alkylating agents resemble those of radiation, these compounds have been examined to learn whether they too are carcinogenic. Several nitrogen mustards (Boyland & Horning, 1949; Heston, 1949, 1953; Griffin et al., 1950; Koller, 1953), sulfur mustard (Heston, 1953), several simple N-acylethyleneimines and some other monofunctional ethyleneimines (Walpole et al., 1954), and a few polyfunctional ethyleneimines including tretamine (Walpole et al., 1954) and busulfan (Koller, 1953) have proved to be carcinogenic. Most investigators found that various sarcomas predominated. Most of the sarcomas occurred at the injection site but some metastasized or were transplantable. A smaller number of adenomas, carcinomas, and other neoplasms have been reported (Boyland & Horning, 1949; Heston, 1949; Walpole et al., 1954), but even many of these were associated with the injection site. In general, maximal sublethal doses are required to produce the effect. It is of interest that some of the carcinogenic monofunctional alkylating agents have no demonstrable tumour-inhibiting action (Walpole et al., 1954). As yet, there is no evidence that any alkylating agent has caused tumours in man (Walpole, 1958). In fact, it appears that no highly active carcinogen, even for animals, has been found among the alkylating agents. Their action tends to be slow. It has been claimed (Griffin et al., 1950) and denied (McDonald, 1961) that a single dose of nitrogen mustard is as effective as multiple doses in promoting the occurrence of neoplasms. In fact, nitrogen mustard may be a cocarcinogen and not a carcinogen at all (McDonald, 1961).

The nature and significance of chromosomal abnormalities in tumours produced in rats by aromatic mustards have been discussed by Koller (1953). In 1950, Philips concluded that the relationship between mutagenicity and carcinogenicity remains to be established, and in spite of very extensive discussion

(Haddow, 1958, 1959) the conclusion remains valid today.

Sterilization

In experimental animals an entire range of sterilizing effects has been produced by alkylating agents. These effects have been most fully explored in the rat. However, the mouse (Cattanach, 1957, 1959a, 1959b; Cattanach & Edwards, 1958), the rabbit (Jackson, 1959; Fox, Jackson et al., 1963) and the dog (Hendry, Homer et al., 1951) as well as insects are susceptible to the sterilizing effects of tretamine. Busulfan and isopropyl methanesulphonate have produced aspermia in rabbits (Fox, Jackson et al., 1963). The lowest effective daily dosage of tretamine tested in the rabbit was 0.01 mg/kg/day intravenously (Jackson, 1959), a dosage of about the same magnitude as that used to treat cancer in man (Sykes et al., 1956).[1] In contrast, a single dose of tretamine at the rate of 0.2 mg/kg intraperitoneally produced subfertility promptly and sterility most marked 22-26 days after the dose and ending about 35 days after the dose (Bock & Jackson, 1957; Craig et al., 1958; Jackson, 1958). The effect was thought to be on spermatocytes; mature sperm present at the time of treatment were not affected (Craig et al., 1958; Jackson, 1959). When the frequency of dosage with tretamine was increased to 0.2 mg/kg/day for five days, intraperitoneally, recovery occurred five to six weeks after treatment (Bock & Jackson, 1957; Craig et al., 1958), but there was apparently a second brief period of sterility about nine weeks after the dose (Craig et al., 1958). Thus, the effect resembled the combined action of single doses of tretamine and busulfan. The first period of sterility was thought to be caused by destruction of spermatocytes, the second by destruction of spermatogonia (Jackson, 1959). However, a quantitative, histological study (Steinberger, 1962) showed that the initial lesion caused by this dosage of tretamine (0.2 mg/kg/day intraperitoneally for five days) involved early generations of type A spermatogonia and began at least as early as the second day after initiation of treatment. A sharp decrease in the number of resting spermatocytes occurred on the tenth day after the start of treatment and this decrease was soon reflected in the more mature spermatocytes. It appears that the effect of such doses on fertility involves a combination of this maturation depletion and the more subtle effect on the more mature germinal cells which is also produced by low doses (Steinberger et al., 1959).

When the same dosage of tretamine (0.2 mg/kg/day) was given a total of 21 times in the course of 57 days, the rats remained sterile for at least eight weeks after dosage was stopped (Jackson & Bock, 1955) and, in fact, only one was fertile 75 days after the last dose (Bock & Jackson, 1957).

Forty-one doses of tretamine at the lower rate of 0.05 mg/kg/day intraperitoneally during 57 days produced complete sterility in male rats beginning a few days after the first dose and lasting for three to four weeks after the last dose, when full recovery took place (Bock & Jackson, 1957; Jackson & Bock, 1955). During the sterile period, the sperm were normal in number and motility and were able to penetrate ova, but the ova failed to develop to a significant degree. The sexual behaviour of the males remained normal (Jackson & Bock, 1955). No histological damage to the testis was observed following a dosage of 0.05 mg/kg five times each week (Jackson & Bock, 1955; Steinberger et al., 1959). The action of tretamine that permits fertilization but leads to early death of the resulting zygotes or embryos involves mature spermatozoa as well as other germinal cells.

When a similar experiment with tretamine (0.05 mg/kg/day intravenously for 29 days) was carried out in rabbits, the sperm were infertile but otherwise normal for about 50 days, after which matings became abruptly aspermic, although the volume of seminal fluid remained normal (Jackson, 1959).

Most of the effects of tretamine on fertility just mentioned were confirmed by Steinberger et al. (1959), who found, in addition, that the F_1 progeny of males that had regained fertility developed normally and were fully fertile, as was the F_2 generation.

Tretamine at a dosage of 0.2 mg/kg/day for five days before mating did not affect the fertility of female rats (Jackson & Bock, 1955), However, a single dose of 1.6 mg/kg did reduce the fertility of female mice (Cattanach, 1959a).

Several of the compounds, including tretamine, produce teratogenic effects or complete abortions when given after fertilization (see above). For tretamine, the required dosage in rats (0.2 mg/kg/day for five days beginning four days after mating) (Jackson & Bock, 1955) was larger than that needed to produce sterility in the male and increasing doses were required as the foetus grew older.

[1] The standard initial testing dose for flies is 5 mg/kg (1 μl of 10% solution per 20-mg fly)—C. N. Smith, personal communication.

The various alkylating agents show a selective effect on the different stages of spermatogenesis. The ethyleneimines may interfere with the function of spermatozoa or spermatids or they may interfere with early development and even lead to atrophy of the seminal epithelium (Jackson et al., 1959). Busulfan and other relatively complex alkane sulfonic esters act on spermatogonia and lead to aspermia. By contrast, certain simple alkane sulfonic esters (methylmethanesulfonate, ethylmethanesulfonate, and CB 1506 (the sixth item in Table 1)) act on spermatids and spermatozoa with no histological or functional evidence of action on earlier stages. The first two compounds produce complete sterility with no loss of libido when given in appropriate dosage by mouth. The sterility is completely reversible. So far no side-effects, including carcinogenesis or mutagenesis, have been found in spite of search (Jackson et al., 1961).

Alkylating agents differ qualitatively in their effect on the mouse testis when judged by production of chromosomal fragments and bridges (Moutschen, 1961).

There is even considerable variation in the ability of alkylating agents to produce sterility of any kind (Jackson et al., 1960). Although the maximal tolerated dose of methyl-bis(β-chloroethyl)amine *N*-oxide produced sterility accompanied by testicular atrophy (Goldeck & Hagenah, 1951), two aromatic nitrogen mustards—melphalan (Jackson, 1958; Jackson et al., 1960) and *p*-(*N*,*N*-di-2-chloroethyl)aminophenylbutyric acid—did not interfere with the fertility of male rats, even when given at dosages that produce transient leukopenia and inhibit tumour growth (Jackson, 1959).

Different alkylating agents vary also in their effects on the sperm and eggs of certain invertebrates, including sea urchins (Fox, Partington & Jackson, 1963).

Carcinostatic effect

Alkylating agents have a firm place in the treatment of many neoplasms. The therapeutic aspects have been reviewed by Sykes and her colleagues (1956) and by Hall (1962).

Different alkylating agents may have different effects on the haematopoietic tissues of the same species. In general, busulfan and similar compounds have their most marked effects on neutrophils and platelets, while the nitrogen mustards affect chiefly the lymphocytes. Each finds use against corresponding tumours—granulocytic leukaemia and lymphomas, respectively. By combining a compound from each series it may be possible to simulate more exactly the effects of radiation (Elson, 1958). The possibility of combining carcinostatic drugs for improved therapy is just beginning to be explored (Ross, 1962).

Quantitative toxicity

It has been suggested by Ross (1962) that the various observed effects characteristic of alkylating agents can be related to dosage and described under three headings representing increasingly severe injury: (1) cytostatic effect in which mitosis of a resting cell is delayed or entirely prevented, (2) mutagenic effect in which division proceeds but the new cells have altered properties, and (3) cytotoxic effect in which damage is so great that the cell dies. Dosage effects may be very precise as long as other conditions of experimentation remain the same. Direct application of sulfur mustard to the eyes of rats at the rate of 0.01 $\mu g/cm^2$ causes only suppression of division of epithelial cells; if the inhibition is continued for a week by daily applications, the tissue as a whole continues to grow at the normal rate but still with no cell division. A dosage of 0.1 $\mu g/cm^2$ results in nuclear fragmentation, and a dosage of 1.0 $\mu g/cm^2$ causes pyknosis of all cells and permanent scarring of the epithelium (Friedenwald et al., 1948).

It is suggested that the action of alkylating agents on primitive blood cells to produce temporary leukopenia or on germ cells to produce temporary sterility are cytostatic effects. Mutagenesis and carcinogenesis are mutagenic effects by definition. Killing of tumour cells or normal differentiated blood cells is obviously cytotoxic. The difficulty is that mature blood cells are often killed and primitive ones spared by the same dose of drug. Whether teratogenesis is caused by the killing of critical cells or merely by interruption of the normal rate of cell division is seldom clear. Under these circumstances, the naming of " processes " may only serve to conceal our ignorance by making the relation between observation and theory seem more substantial than it really is.

Early studies reviewed by Philips (1950) showed that a mustard gas has the same order of toxicity for different species when it is given intravenously, but that the species variation is considerably greater for dermal toxicity although the amount of a mustard gas necessary to produce damage is extremely small. Total absorption of as little as 6 $\mu g/cm^2$ of sulfur mustard or tris(2-chloroethyl)amine causes

vesication of 50% of white human subjects (Nagy et al., 1946). The unreacted vapour disappears within a few minutes and the amount of vesication is proportional to the amount fixed in the tissue (Philips, 1950).

The toxicity of some candidate chemosterilants, several of which are also anticancer drugs, is shown in Table 3. Some of these compounds have a moderate acute toxicity compared with other pesticides while tretamine has a high toxicity similar to that of mustard gas or the insecticide tetraethylpyrophosphate. Animals may withstand daily feeding of only a relatively small proportion of the LD_{50} dosage of the candidate chemosterilants without clinical effect or leukopenia. On the contrary, the dosage necessary to control tumour growth is often one that may injure the haematopoietic system.

From a clinical standpoint, it is usually the effect on bone marrow that limits the dosage of alkylating agents used to treat cancer (Sternberg et al., 1958). However, it is a common finding that animals or people may recover completely following a single or repeated dosage sufficient to produce a marked reduction of circulating white blood cells. Although this is a convenient and customary basis for monitor-

TABLE 3
TOXICITY OF CHEMOSTERILANTS

Compound	Toxic dosage in rats				Approximate therapeutic dosage in man		
	Single dosage		Repeated daily dosage				
	Oral LD_{50} (mg/kg)	Dermal LD_{50} (mg/kg)	mg/kg/day	Effect	mg/kg	Doses	Route
Metepa [a]	136	183	10.0 (×104; oral)	Reduced white blood cells, growth suppression and death in 44-94 days	Not used		
			5.0 (×104, oral)	Sterility, testicular atrophy and 1 of 12 died in 89 days			
			2.5 (×197; oral)	Subfertility and partial testicular atrophy			
			1.25 (×197; oral)	None			
Tepa	37 [a]	87 [a]			1.2-2.0	Total	Intramuscular [b]
Apholate	98 [a] 90 [d]	400-800 [a]	15.0 (×98; in food)	Reduced white blood cells, growth suppression, testicular atrophy and 1 of 5 died in 49 days [c]	0.6	3-6	Oral [d]
			7.5 (×98; in food)	Growth suppression and testicular atrophy [c]			
			3.7 (×77; in food)	Growth suppression and testicular atrophy [c]			
Tretamine	ca. 1		0.2 (×1; intraperiton.)	Temporary sterility [e]	0.12	Total	Intravenous [b]
			0.2 (×21; intraperiton.)	Continuing sterility [f]	0.04-0.08	4	Oral [g]
			0.05 (×41; intraperiton.)	Temporary sterility, and without atrophy [e]	0.3-0.6	Total	Oral [b]

[a] Gaines & Kimbrough (1964); see the article on page 737 of this issue.
[b] Sykes et al. (1956).
[c] Gaines, T. B.—unpublished data.
[d] Olin Mathieson Chemical Co.
[e] Craig et al. (1958).
[f] Jackson & Bock (1955).
[g] Hall (1962).

ing people with occupational exposure to the compounds, Table 3 shows that testicular atrophy and failure to gain weight at a normal rate may be more sensitive measures of injury in the rat. Other, more sensitive criteria may be found as attention is turned to the long-term effects of low dosages.

Furthermore, species differ in sensitivity. Calves were killed by a single intramuscular dose of apholate at the rate of 2.5 mg/kg (Khan, 1963). All sheep were killed by apholate or metepa at a daily oral dosage of 2 mg/kg (Younger & Radeleff, 1964), although either compound produced inconspicuous injury to rats when given in slightly larger dosages for longer periods (see Table 3). The sheep showed focal necrosis and other lesions of the liver, kidneys and lungs (Younger & Radeleff, 1964). Whether these lesions, which are unusual for the ethyleneimines, reflect a mode of action peculiar to sheep is not clear.

Distribution, storage, and fate

When mustards are injected intravenously, they are eliminated from the blood and fixed within a few minutes. Cross-circulation of the blood of dogs begun as early as 30 minutes after intoxication and maintained for 45-180 minutes permitted the nonpoisoned member to survive without untoward effect even though the other dog had received enough sulfur mustard intraperitoneally to cause death in five hours (or death of both dogs in other tests where cross-circulation was present at the time of injecting the dose) (Black & Thomson, 1946). Occlusion of the blood supply of the legs of rats for less than 15 minutes after a dose of the same drug or temporary occlusion of the arterial supply of the intestine protected the bone marrow or intestinal epithelium distal to the clamp but not the life of the animal (Karnofsky et al., 1948).

Rapid disappearance from the blood has also been demonstrated by measurement of activity of samples following both intravenous and oral doses of radioactive tretamine, tepa, and busulfan in man. The proportion of the intravenously injected dose in the blood falls to less than 5% within 30 minutes and then remains essentially stable for at least 48 hours. Approximately the same proportion of an oral dose is reached in the blood, beginning about four hours after the dose (Nadkarni et al., 1959). However, it seems likely that most, if not all, of the activity is from metabolites soon after a dose by either route.

Using analytical methods specific for the two drugs, Mellett & Woods (1960) found that when thiotepa was given to dogs either intravenously or orally it disappeared rapidly from the plasma, but the tepa metabolized from it reached a higher concentration and persisted longer. Thus the concentration of tepa at four hours was as great as or greater than the concentration of thiotepa at two hours. At least 50% of the oral dose of thiotepa was absorbed intact.

From a chemical standpoint, the alkylating agents are moderately to highly reactive. Tepa is explosive under certain conditions. Since enzymes attack some molecules that are chemically quite inert, one would expect all alkylating agents to be actively and rapidly metabolized. Apparently metabolism is rapid and essentially complete in some instances but limited in others. Species may differ widely in the degree to which they metabolize the same compound even though no significant difference may be apparent between the toxic action of the compound in one species and that in another. Some metabolism may be extraneous to toxicity, and species that rapidly excrete a high proportion of unreacted drug may hold the key to understanding *essential* reactions. Thus, Craig & Jackson (1955) suggested that the cytotoxic activity of tepa may be associated with interference by a very small proportion of the dose with some highly susceptible cellular mechanism.

Using isotopically marked tepa and paper chromatography, no unchanged tepa was detected in the urine of mice, and 80% was degraded to the inorganic phosphate. The animals excreted 60%-80% of the radioactive phosphorus from tepa in 24 hours (Nadkarni et al., 1957). No unchanged tepa was excreted in human urine, but only about 30% of the dose was recovered (Nadkarni et al., 1959). Of the recovered material, 88% or more of the radioactivity was present as inorganic phosphate (Nadkarni et al., 1959). A high proportion of tepa can be recovered from the urine of rats (Crossley et al., 1953; Craig & Jackson, 1955); however, the majority is in the form of unchanged drug (Craig & Jackson, 1955). Thus, tepa is not highly reactive in the rat although a small fraction of the dose is probably metabolized extensively. In the dog, 13%-15% of oral doses were recovered unchanged in the urine (Mellett & Woods, 1960).

Mice are moderately efficient in metabolizing metepa. In an experiment in which 65% of the dose of metepa was recovered in the urine, only a little over 16% of the total dose was excreted unchanged (Plapp et al., 1962).

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Using isotopically marked tepa and paper chromatography, no unchanged tepa was detected in the urine of mice, and 80% was degraded to the inorganic phosphate. The animals excreted 60%-80% of the radioactive phosphorus from tepa in 24 hours (Nadkarni et al., 1957). No unchanged tepa was excreted in human urine, but only about 30% of the dose was recovered (Nadkarni et al., 1959). Of the recovered material, 88% or more of the radioactivity was present as inorganic phosphate (Nadkarni et al., 1959). A high proportion of tepa can be recovered from the urine of rats (Crossley et al., 1953; Craig & Jackson, 1955); however, the majority is in the form of unchanged drug (Craig & Jackson, 1955). Thus, tepa is not highly reactive in the rat although a small fraction of the dose is probably metabolized extensively. In the dog, 13%-15% of oral doses were recovered unchanged in the urine (Mellett & Woods, 1960).

Mice are moderately efficient in metabolizing metepa. In an experiment in which 65% of the dose of metepa was recovered in the urine, only a little over 16% of the total dose was excreted unchanged (Plapp et al., 1962).

The metabolism of thiotepa is similar to that of tepa in rats. This species excreted at least 95% of the drug and its metabolites within eight hours of intravenous or intra-arterial injection. The remainder was excreted about equally in the faeces and expired air. Between 70% and 80% of the drug in the urine was unreacted, while 20%-30% was in the form of tepa. There was evidence of traces of two or three other unidentified metabolites (Boone et al., 1962). In the rat, about 2% of injected thiotepa is expired as carbon dioxide (Boone et al., 1962) and this may come from the 20%-30% metabolized to tepa. Dogs excrete 0.3%-0.7% of thiotepa in the urine unchanged and 8%-15% as tepa (Mellett & Woods, 1960). Craig et al. (1959) studied the metabolism of thiotepa in the mouse, rat, rabbit and dog in such a way that the results were strictly comparable. Each species converted the compound to tepa and each produced some inorganic phosphate and at least traces of other metabolites; however, the details of the biotransformation were not the same either quantitatively or qualitatively in any two of the species.

Following intravenous injection in man and animals, tretamine decreased rapidly in the blood and then the radioactivity maintained a low constant level in the blood for at least 48 hours. Mice excreted between 72% and 88% of radioactive tretamine in 24 hours, mostly in the urine but 1%-2% as exhaled carbon dioxide. Humans excreted about 30% in the urine after an oral dose but only about 20% after an intravenous dose. There was no unchanged compound in the urine. Chromatography revealed at least 16 metabolites in the urine, one of which is cyanuric acid. The metabolic pathway is unknown, but it has been postulated that the ethyleneimino group is excreted as the alkylated derivatives of normal metabolites (Nadkarni et al., 1959; Smith et al., 1958).

Radioactive morzid was metabolized in both rats and man to phosphate and morpholine, and the sulfur was oxidized to form *N*-(3-oxapentamethylene)-*N'*,*N''*-diethylenephosphoramide, a compound of equal potency against a test tumour. In addition, man excreted a considerable amount of uncharacterized metabolites (Heidelberger & Maller, 1958).

A smaller proportion of the radioactivity was recovered in the urine of cancer patients treated with ^{14}C-busulfan (about 30%) than with ^{35}S-busulfan (about 45%-60%). Methane-sulfonic acid accounted for 95% of the excreted activity derived from sulfur. On the contrary 12 radioactive materials were separated by ion exchange from the carbon-tagged drug. No unchanged drug was recovered from the urine (Nadkarni et al., 1959).

Rats injected with carbon-labelled ethylmethanesulfonate exhaled 25%-35% as carbon dioxide in 24 hours; only 5%-8% remained in the body (Roberts, 1958), and it may have been metabolized.

Biochemical action and selectivity

Some enzymes are inhibited *in vitro* but not at concentrations below 10^{-4} molar, whereas equal distribution of a median lethal dose of methyl-bis (2-chloroethyl)amine, for example, would result in a concentration of only 10^{-5} molar in the body (Philips, 1950). The problem of interpretation is not confined to enzymes. Mandel (1959) stated: "The nonspecific *in vitro* reactions of the agents with amino acids, purines, pyrimidines, proteins, RNA, DNA, and many other compounds have been most controversial in the elucidation of the *in vivo* action, largely because such enormous doses were usually used in order to provoke the desired response, and physiological conditions were not duplicated".

Some investigators satisfied themselves from *in vitro* studies that, while reactions with amino and sulfhydryl groups occur, reaction with carboxyl groups in proteins and reaction with phosphate groups in nucleic acids are most characteristic (Alexander & Stacey, 1958; Stacey et al., 1958). The same authors concluded that the polyfunctional character of the carcinostatic compounds enabled them to produce cross-links in the same molecule or between molecules which they considered the most efficient way of altering the function of DNA and similar molecules. They showed electron micrographs ostensibly of cross-linked DNA (Stacey et al., 1958). By contrast, genetic studies mentioned earlier showed that polyfunctional alkylating agents were relatively ineffective as mutagens and that the changes that do occur in living DNA are not brought about by cross-linkage (Auerbach, 1958). Papirmeister (1961) has attempted a chemical explanation of this observed difference, between mono- and polyfunctional alkylating agents.

Traces of the radioactivity from the ethyl moiety of tagged ethylmethanesulfonate were associated in the urine with the amino acids serine, alanine, and arginine, and with urea; however, the majority was associated with *N*-acetyl-*S*-ethyl cysteine, and a related compound. This was considered the first *in vivo* evidence of plain alkylation in the case of a

biologically active alkylating agent (Roberts, 1958). The finding is consistent with the conclusion from organic chemistry that, in a wide variety of reactions, alkylation proceeds preferentially on sulfur, then nitrogen, and least readily on oxygen (Price, 1958). However, partly because of the rarity of such findings, Elson (1958) has suggested that a typical compound does not exert its biological action through an alkylating mechanism at all.

In 1951 Butler et al. (1951) suggested that esterification of phosphate groups might be an important reaction of alkylating agents with nucleic acids, but Brookes & Lawley (1960) failed to find evidence of such a reaction in experiments designed to find the most reactive centres. Instead, they found that the only detectable reaction with RNA and DNA both *in vitro* and *in vivo* was at N-7 of guanine moieties. Later a second reactive site accounting for about 10% of the total alkylation was found at N-3 of adenine (Lawley & Brookes, 1963).

The chemical mechanism of action is far from agreed on. It may be that a very wide range of cell constituents are reactive (Philips, 1950; Ross, 1958). The interaction of particularly active enzyme systems, direct reactions with -SH groups, changes in the amino acid code of proteins, and influence on cell permeability, as well as direct action on DNA have been discussed (Ross, 1962). The present tendency is to emphasize the importance of action on DNA (Biesele, 1963; Brookes & Lawley, 1964; Papirmeister, 1961; Wheeler, 1962), although the possible importance of other reactions is acknowledged (Biesele, 1963; Brookes & Lawley, 1964), especially in connexion with acute effects (Wheeler, 1962).

Some evidence has been presented that ionizing radiations and alkylating agents both act primarily through an attack on DNA (Philips, 1950) but by different chemical reactions (Alexander & Stacey, 1958). In fact, it has been proposed that radiation, carcinogenic chemicals, and cancer viruses produce the same basic change (Horsfall, 1962). Even if these broad generalities were eventually proved, the observed quantitative (if not qualitative) variation between the effects of different alkylating agents and between different groups of these agents would remain. This variation is what one would expect. In order to undergo a critical reaction, a chemical or its active metabolite must escape destructive metabolism until it reaches the exact site of action with the right chemical and steric configuration still intact.

There is a tendency to lump all alkylating agents together and assume that their properties are essentially identical. Several illustrations disproving this assumption have been cited in the preceding paragraphs. Ross (1962) devoted an entire chapter of his recent book to evidence of selectivity of action, and such action has been noted by others (Mandel, 1959).

Selectivity may involve different species or different tissues in the same organism. There is a lack of a dependable relationship between effects on germinal epithelium, haematopoetic tissues, and tumours (Jackson et al., 1959). The biochemical basis of selectivity is not understood. Distribution of alkylating agents is relatively uniform (Craig & Jackson, 1955; Crossley et al., 1953; Plapp et al., 1962) and is frequently as high or higher to the lung, liver, and kidney as to sensitive tissues (Boone et al., 1962; Craig et al., 1959; Smith et al., 1958), so that selective distribution does not appear to be the cause of selective action. Bone marrow may be a partial exception (Mellett & Woods, 1960). In any event, none of the proposed mechanisms of action has been correlated quantitatively with cytotoxic effects. Cells that are naturally relatively immune or that have acquired resistance may show the same biochemical reactions as susceptible cells. Thus, the possibility must be considered that even the first or most sensitive biochemical effect observed so far is merely an epiphenomenon and that the real biochemical mode of action remains undiscovered. Wheeler (1962) made these observations with respect to the anticancer properties of alkylating compounds, but they apply to other pharmacological effects also.

Selectivity of action may be evident within the same cell. Thus synthesis of RNA may continue at a normal rate at concentrations of methyl-bis (2-chloroethyl)amine sufficient to inhibit both synthesis of DNA and cell reproduction (Bodenstein & Kondritzer, 1948). When bacterial cells have been treated with alkylating agents and subsequently infected with bacteriophages, which are largely DNA and proteins, multiplication of the viruses is supported even though proliferation of the cells is, of course, prevented (Herriott & Price, 1948). This situation indicates an extremely delicate and specific change in the cell.

Another evidence of delicate balance is that tumours treated with alkylating agents acquire an immunity so frequently that the phenomenon is taken for granted in therapy. Although this fact is seldom

discussed in connexion with the basic mode of action of the compounds, it would have to be taken into account in any really comprehensive theory of action. There is no recognized relation between acquired tolerance and inheritable resistance. It is not known whether strains of animals could develop resistance to an alkylating agent.

Whether current theories are gradually refined or have to be severely modified, the fact remains that alkylating agents—not to mention antimetabolites, alkaloids, and other compounds—offer some chance of selective action that may be exploited in developing a practical chemosterilant. Particular attention has been focused recently on certain analogues of ethyleneimines that are effective chemosterilants of insects although it is said that they are not lakylating agents (Chang et al., 1964). However, the possibility that the analogues can produce sexual sterility in mammals cannot be excluded without study. It has already been shown that a closely related compound (trimethylolmelamine) is a mutagen in *Drosophila* (Röhrborn, 1962) and causes testicular atrophy in the dog (Hendry, Rose & Walpole, 1951). *N,N*-Dimethylstearamide and some related compounds are carcinogenic (Walpole et al., 1954). Thus, each compound will have to be judged on its own merits with due attention to its particular dosage-response relationships.

RÉSUMÉ

Parmi les moyens utilisés pour lutter contre les insectes, et sans doute aussi contre d'autres espèces nuisibles, les procédés basés sur la stérilisation des individus mâles peuvent se révéler plus efficaces que les méthodes de destruction devenues classiques. L'auteur rappelle que les rayonnements ionisants ont été employés dans ce but aux Etats-Unis et qu'ils ont permis d'obtenir l'éradication complète de la lucilie bouchère.

A cet égard, les propriétés de certains composés chimiques apparaissent également pleines de promesses: ils seraient capables de provoquer la stérilité chez les insectes, sans avoir les inconvénients d'ordre pratique inhérents à l'utilisation des rayonnements. Les plus importants de ces stérilisants chimiques sont les agents alcoylants: moutardes au soufre, moutardes à l'azote, composés époxy, β-lactones, et surtout les éthylène-imines et les esters de l'acide méthane-sulfonique. Après un bref historique, l'auteur, se référant à une abondante bibliographie, fait une mise au point des particularités d'action et de la toxicologie de ces produits.

Les agents alcoylants ont une activité pharmacologique immédiate assez limitée, mais tous sont remarquables par leur action sélective sur les tissus hématopoiétiques et certains tissus doués d'un pouvoir de prolifération élevé. Plusieurs ont un effet mutagène sur les insectes, les bactéries, les champignons et les végétaux plus évolués; un effet carcinogène chez les mammifères, et une action tératogène chez les insectes, les oiseaux et les mammifères. Chez ces derniers, on a mentionné des cas de stérilité complète ou partielle dans la descendance, mais, apparemment, aucun cas de transmission d'une anomalie morphologique provoquée chimiquement. Certains agents alcoylants peuvent causer des difformités chez les embryons de mammifères, mais à des doses relativement élevées. On n'a jamais démontré, par ailleurs, que l'un ou l'autre de ces composés soit à l'origine de tumeurs chez l'homme et en fait, parmi ce groupe, on ne trouve aucun produit ayant une action carcinogène vraiment marquée, même chez les animaux.

Certains de ces agents provoquent la stérilité et, chez les mammifères et les insectes, ce résultat s'observe même si les doses utilisées sont impuissantes à déterminer d'autres réactions. D'autres produits du même groupe, en revanche, n'ont aucune influence sur la fertilité à des doses qui cependant amènent une leucopénie transitoire et l'inhibition de la croissance tumorale. La stérilité peut résulter d'une atrophie testiculaire, d'effets sélectifs sur un ou plusieurs stades de la spermatogenèse, ou encore d'une modification de nature inconnue des qualités du sperme: celui-ci reste abondant, ses éléments sont toujours mobiles et capables de pénétrer dans l'ovule, mais ils ne peuvent assurer un développement notable de ce dernier.

A un premier examen, l'action de ces composés apparaît donc comme étant très voisine de celle produite par les rayonnements ionisants, mais une étude plus approfondie montre des différences quantitatives et qualitatives. Même si les uns et les autres attaquent l'ADN, les réactions chimiques en cause ne sont pas identiques et les modalités d'absorption, de répartition et de réaction en un endroit déterminé sont vraisemblablement différentes dans les deux cas. De plus, les agents alcoylants réagissent non seulement avec l'ADN, mais aussi avec d'autres constituants de la cellule. L'action chimique qui est à la base de leurs propriétés pharmacologiques n'est pas encore entièrement élucidée.

Par rapport aux insecticides ordinaires, les produits chimiques qui pourraient être utilisés éventuellement comme stérilisants possèdent un degré de toxicité élevé ou moyen. Ils sont métabolisés et excrétés rapidement, de sorte que leur accumulation dans l'organisme est minime. Il reste à savoir s'ils auront un effet cumulatif chez les mammifères exposés à en absorber une certaine quantité.

Etant donné que les agents alcoylants peuvent présenter une spécificité marquée pour différents tissus et même pour divers stades de la spermatogenèse, il convient d'étudier la toxicologie de chacun d'eux séparément.

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EFFECT OF DDT ON ANTIBODY RESPONSE TO TYPHOID VACCINE IN RABBITS AND MAN

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J. Shiplov(1), C. D.Graber(2),J.E.Keil, and S. H. Sandifer
Departments of Microbiology and Preventive Medicine
Medical University of South Carolina
Charleston, South Carolina

Abstract

DDT-dosed humans and animals injected with Salmonella typhi vaccine produced O, H and Vi agglutinin titers somewhat higher than controls (no DDT). This is in contrast to an earlier report that DDT-fed animals injected with this vaccine experienced a 30% decrease in antibody titre to whole organisms. Immunoglobulin levels in human volunteers were unaffected by DDT ingestion.

Reports of the effect of DDT on the immune response of animals and man are sparse. Askari and Gabliks (3) showed reduced severity of anaphylaxis evoked with diphtheria toxoid in DDT-fed guinea pigs. Nedopriadko et al (4) demonstrated heightened delayed sensitivity over controls in BCG-sensitized guinea pigs given a single homeopathic dose of DDT. Kotlarek-Haus et al (5) described autoimmune hemolytic anemia in a Polish farmer believed due to DDT sensitization. Wassermann et al injected DDT-dosed rats and rabbits (6,7) with soluble and particulate antigens and reported approximately a 30% decrease in mean antibody titre to both immunogens.

Results reported here of the effect of DDT on both the primary and secondary responses in rabbits injected with S. typhi vaccine are at variance with the Wassermann findings. Also reported is the effect of DDT on the anamnestic response in humans to S.typhi vaccine, the first study of this kind that we are aware of.

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Materials and Methods

Human Study

Seven healthy volunteers (ranging from 30-55 years) all recently immunized with S. typhi vaccine were used for this study. Physical examinations were performed weekly on the three individuals, who were given a daily oral dose of 5 mg of DDT(8) for 20 days. On day 31 all seven were injected intradermally with 0.1 ml of S. typhi vaccine (Lederle, Pearl River, N.Y.). Serum from blood samples taken on day 0 (baseline) and at days 37, 45, 52 and 59 was preserved at -20C to await serological studies of Vi, H and O agglutinins. Immunoglobulin levels were determined by the Mancini technique (9) for IgG, IgM, IgA on baseline sera and weekly specimens thereafter for eight weeks and reported as geometric mean levels. DDT levels determined by gas chromatography were made shortly before DDT ingestion and prior to S. typhi vaccine injection.

Animal Study

Thirty male, New Zealand white rabbits (ca. 2.3 Kg) were divided into 4 groups: Group A (12 rabbits) and Group B (6 rabbits) were inoculated intravenously on day 5 of the experiment with 1.0 ml of S. typhi vaccine to evoke a primary response and on day 45 with 2.0 ml of the vaccine to elicit an anamnestic response. Group A rabbits received 10 mg DDT per day (human formulation) from day one through day 10 by force-feeding with an animal feeding tube fitted to a 30 ml syringe. Group C (6 rabbits) were similarly force-fed DDT but given no vaccine. Group B rabbits were given no DDT. Group D (6 rabbits) received no vaccine or DDT and served as normal controls. Normal rabbit diet was provided for all animal groups. Blood was drawn at the beginning of the experiment (baseline), on day 15 to measure primary response to immunogen and on day 49 to measure secondary response. DDD levels were

assessed by gas chromatography at each blood acquisition. Weight of all animals was recorded weekly.

Serology

An aqueous extract of Vi material from opaque colonies of Citrobacter ballerup was prepared to yield an antigen which was used at a concentration of 100 gamma per milliliter to sensitize fresh sheep red cells (SRBC). A one percent suspension of Vi-sensitized sheep red cells was used to test human and animal sera for Vi agglutinins.

O antigen (IX, XII) and H antigen (d) from S. typhi (Difco Co., Detroit, Michigan), used for routine detection of febrile agglutinins to this organism, were employed for measuring O and H agglutinins in humans and rabbits. Appropriate controls (sensitized red cells plus positive and negative sera, O and H antigen plus positive and negative sera) were included for all three tests.

Results

Human Study

All three individuals ingesting DDT suffered no discernible ill effects during the study and have remained healthy to date. Periodic physical examinations conducted by our physician team member (SHS) revealed no weight loss or any changes in any measurable physiologic parameters. DDT-dosed individuals showed a rise in mean serum levels from 10.1 parts per billion (ppb) at day 0 to 22.1 ppb for DDT at day 21 when the dosage was discontinued. These levels have subsequently returned to baseline figures.

Table I shows the serology for the three antigens used to measure the anamnestic response in the seven individuals tested.

TABLE I

Geometric Mean Titre and Range for O, H Bacterial Agglutinins and Vi Hemagglutinins in DDT-Dosed and Non-Dosed Humans Receiving S. typhi Vaccine to Promote Anamnestic Response

Antigen Measured:	Group	Sampling Day 0*	37	45	52	59
O	Test (DDT & Antigen)	3** (2-4)	40 (16-64)	13 (4-32)	16 (4-64)	20 (4-64)
	Control (Antigen only)	5 (2-16)	9 (2-32)	9 (2-16)	6 (4-16)	11 (8-16)
H	T	20 (16-32)	40 (32-64)	25 (16-64)	40 (32-64)	40 (32-64)
	C	8 (2-16)	14 (2-16)	19 (16-32)	13 (8-32)	23 (16-32)
Vi	T	2 (1-4)	6 (4-12)	10 (4-32)	6 (4-8)	5 (4-12)
	C	2 (1-4)	5 (1-16)	4 (1-16)	5 (1-8)	5 (1-8)

*DDT administered on days 0 thru 20. S.typhi Vaccine given on day 31.
**Geometric mean.

Geometric mean titre (GMT) of O antibody in the three DDT-dosed individuals showed a 13-fold rise over baseline levels at 1 week and an eightfold rise over the control group for this period. Thereafter up to day 59, approximate doubling of control values by the test group occurred.

The H titre antibody levels were three times those of controls at seven days and remained approximately twice the controls through day 59. A proportionate rise in GMT for both test and control groups occurred.

Vi hemagglutinins showed generally higher GMT over controls throughout the study, except that by day 59, the test titre was about equal in both cohorts.

Immunoglobulins (IgG, IgM, IgA) levels (Figure 1) for both test and control groups were essentially normal throughout the study period. Geometric mean levels for IgG reached a high normal (1200mg/%) during the first week following the vaccine injection in the DDT-dosed individuals but thereafter were almost identical with control levels.

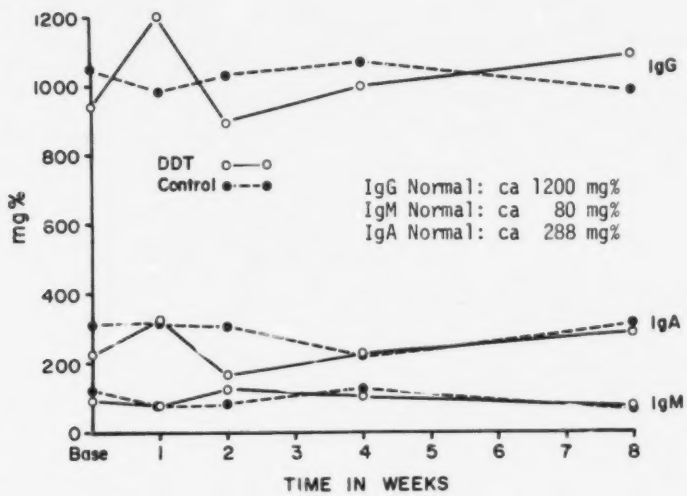

FIGURE I

Major Immunoglobulin Levels in DDT-Dosed and Control Humans

Animal Study

One rabbit from Group A (DDT plus vaccine) died during the study. This occurred immediately after the animal received its scheduled last dosage of DDT. All other animals were healthy and gained weight during the study. Levels of DDT rose in the dosed rabbits from none detectable at day 0 to 54 ppb (Group A) and 73 ppb (Group C) by day 15, five days after DDT dosage was stopped. By day 49, DDT levels had receded to approximately 5 ppb for both groups. No O, H or Vi agglutinins were demonstrable in baseline sera of any rabbits.

Table II shows levels of these agglutinins, measured as primary response to S. typhi vaccine in Group A and Group B rabbits, at day 15, ten days fol-

TABLE II

Animal Distribution by Primary Response* Titres of O-F Bacterial Agglutinins and Vi Hemagglutinins in Group A and Group B Rabbits Given S. typhi Vaccine

Antigen Measured:	Group	Titre					
		128	256	512	1024	Mean	GMT
O	A(11**) DDT & Antigen	2	6	3	3	303	273
	B(6) Antigen	0	4	0	2	512	406
H	A	3	8	0	0	222	210
	B	4	2	0	0	170	162
		No titre	2	4	8	16	Mean	GMT
Vi	A	6	2	2	1	0	2	2
	B	3	0	0	3	0	4	3

*10 days post inoculation (day 15)
**One animal death in this group.
Group C, D animals (controls) received no vaccine and demonstrated no antibody levels to O, H or Vi antigens.

lowing S. typhi administration. In the Group A animals, a GMT of 273 for O antibody occurred as opposed to a GMT of 406 for O antibody in control animals. GMT titres for the primary response to H antigen were 210 for DDT rabbits; 162 for controls. Eight of 11 test rabbits had peak titres of 256 as compared with two of six controls with this titre. The GMT for H antibody was approximately 1.3 times that of controls.

Vi primary response titres were negligible for the two groups receiving the vaccine. Six of 11 test animals showed no titres at all, while three of the six in the control group were unresponsive to Vi.

Table III shows anamnestic antibody titres to S. typhi vaccine as measured with O, H and Vi antigens.

TABLE III

Animal Distribution by Anamnestic Response* Titres of O-H Bacterial Agglutinins and Vi Hemagglutinins in Group A and Group B Rabbits Given S. typhi Vaccine

Antigen Measured:	Group	Titre					
		128	256	512	1024	Mean	GMT
O	A(11**)DDT & Antigen	0	1	7	3	628	585
	B(6) Antigen Only	0	4	2	0	341	323
H	A	0	7	4	0	349	329
	B	2	4	0	0	213	203
	No titre	2	4	8	16	Mean	GMT
Vi	A	2	1	0	1	2	2
	B	2	2	1	0	3	3

*4 days post inoculation (Day 49)
**One animal death in this group.
Group C, 6 animals received no vaccine and demonstrated no antibody levels to O, H or Vi antigens.

The anamnestic response to the vaccine in DDT-dosed rabbits as measured by O agglutination was higher than controls (GMT 585-323). Three test animals had titres of 1024; seven had titres of 512. Two control animals had titres of 512, and four had titres of 256.

Anamnestic agglutinins to H antigen in test animals were higher than those in control rabbits (GMT 329-203). Peak titre for test group was 512, with 4 of eleven animals recorded at this level. Peak titre for control animals was 256.

Vi agglutinins as measured for secondary response were essentially unchanged from those for the primary response. Sera of seven of the test rabbits demonstrated no antibody to the Vi component, while control group sera response to Vi was also minimal.

Discussion

This limited study would seem to indicate that there is little or no evidence that DDT adversely affects the host's humoral response in man or rabbits. In only one instance, were data accrued to support Wassermann's animal findings (6,7). In our animal study, Group B rabbits (vaccine only) produced a higher primary response of O antibody than the Group A animals (DDT plus vaccine). However, by the end of seven weeks, the anamnestic response for the Group A animals to O antigen exceeded that of the controls. The GMT for DDT-dosed animals was almost twice that of animals receiving antigen only. Similarly, anamnestic response to H antigen figured as GMT for DDT-dosed animals was one and one half that of controls. It is conceded that these results may be partly attributable to normal variations in technique.

It is only at the end of the animal experiment that we can compare our findings with those of the Wassermanns, since they measured total response to the whole organism at the end of a series of vaccine injections. They found a 30% reduction in titres to S. typhi vaccine in DDT-dosed rabbits as opposed to

those receiving vaccine only. Our study shows a substantial and possibly significant increase in geometric mean antibody titres to both O and H antigen measured at the experiment's end for test animals over controls.

The human experiment, although limited to three DDT-imbibing humans and measuring only anamnestic response, agrees substantially with the animal findings. The GMT of the three DDT-dosed humans was higher than the four control subjects receiving vaccine only as measured by O antigen. H antibody measurements were similarly higher for DDT-dosed humans than controls, although increase in titre was proportionately the same since the test group had higher baseline values. No explanation is proffered for the biphasic profile of our mean O and H titres of test subjects, although these findings are somewhat in agreement with those of Wassermann (7).

Immunoglobulin levels in humans stayed well within normal limits and except for an initial peaking in IgG for the test group did not vary during the experiment. This is at variance to findings of the Wassermanns for 7S globulin in rabbits and in rats in which they showed a substantial drop in these levels. Wassermann et al measured serum proteins in their animals with disc electrophoresis; our testing of human sera was done by immunodiffusion.

The human experiment, although necessarily limited by numbers, would appear to be more instructive than the Wassermann study in animals. Their animals were given DDT via water taken ad libitum, a condition that may have resulted in dosed animals having DDT levels far in excess of those encountered in nature and evidenced by several deaths. Our three volunteers received 30 times the inadvertent dose of DDT and expressed no symptoms. Our animals received from 50 to 75 times background levels (trace or non-detectable) with no apparent toxicity although electron microscopic studies of the liver of sacrificed animals (not reported here) did show an increase in smooth endoplasmic reticulum. Neither animals or humans in our study demonstrated a

reduced ability to produce humoral antibody. The Vi response was minimal for both animals and humans, but this seems referable to the small amount of solubilized Vi component in the vaccine.

Obviously more work is indicated to study response to antigen in DDT-dosed animals and possibly humans. Interesting would be the effect of DDT on cellular immunity and the fate of certain in vitro correlates of delayed sensitivity (Migration Inhibition Factor, Lymphocyte Transforming Agent, and Lymphocyte Toxicity) under these conditions. Such a study in humans is planned by us.

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DDT AND POLYCHLORINATED BIPHENYL (AROCLOR 1242®) EFFECTS OF UPTAKE ON *E. COLI* GROWTH

J. E. KEIL* and S. H. SANDIFER

Section of Preventive Medicine, Medical University of South Carolina

C. D. GRABER

Microbiology Department, Medical University of South Carolina

and

LAMAR E. PRIESTER

Environmental Health Laboratories, State Board of Health, Columbia, South Carolina

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Abstract—DDT at 0.01 and PCB at 0.01 and 0.1 ppm consistently stimulated *E. coli* growth *in vitro*. Differences between treatments and controls were statistically significant at the 95 per cent confidence level.

E. coli uniformly converted DDT to DDD (75 per cent) and DDE (25 per cent).

There was no difference in nucleic acids content between treatments and controls at the conclusion of 24 hours incubation although increased uridine uptake was noted in all DDT and PCB cultures after 5 h of incubation.

INTRODUCTION

DDT AND polychlorinated biphenyls (PCBs), similar to each other in structure and physiological effects (LICHTENSTEIN *et al.*, 1969), are of concern to the scientific community because of their ubiquity (RISEBOROUGH *et al.*, 1968) and persistence (WURSTER and WINGATE, 1968). The alleged lack of biodegradability of these environmental pollutants has caused alarm and bans of these useful chemicals. Work as early as 1963 by COPE and SANDERS indicated that DDT levels could be significantly lowered by several species of bacteria. Subsequently, other workers (CHACKO, LOCKWOOD and ZABIK, 1966; STENEVSEN, 1965; WEDEMEYER, 1966) reported degradation of DDT by a variety of bacteria and fungi. KEIL's work (1969) also pointed to the metabolism of this insecticide by marine diatoms. With the thesis established that DDT was indeed biodegradable, the authors postulated that human flora may play an important part in the metabolism of DDT, PCB and other ecological contaminants.

Research was performed to study the effects of these chemicals *in vitro* on a facultative organism, common to human intestinal flora, that is the prime indicator of fecal contamination.

MATERIALS AND METHODS

The study organism, *Escherichia coli*, was selected because it is a common autochthonous, human intestinal aerobe, easily cultured, and is an accepted standard indicator of fecal contamination.

Initial work screened a wide range (0–1000 ppm) of DDT and PCB concentration, against *E. coli* by use of impregnated paper filter discs similar to those used to test antibiotic sensitivity. Impregnated discs with DDT and PCB concentrations of 0,

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* JULIAN E. KEIL, M.S., Associate in Preventive Medicine, Medical University of South Carolina, 80 Barre Street, Charleston, South Carolina, 29401, U.S.A.

0.001, 0.01, 0.1, 10, 100, and 1000 ppm were placed on trypticase soy broth (TSB) agar plates which had been inoculated with *E. coli*. Experimentation indicated that 6 mm diameter discs cut from No. 2 Wattman filter paper would absorb 0.02 ml of the acetone solvent. The discs were dried prior to use.

For the first experimental series (I) twenty-eight 1-l. Erlenmeyer flasks, each with 500 ml TSB, were inoculated with *E. coli*. Four flasks served as controls, four for each of two levels of DDT and PCB treatments, and four as acetone controls since acetone was the suspending vehicle for the toxicants. Additionally, four flasks were used as uridine controls. One flask, containing only media, was used as a growth check. Each flask was vigorously agitated every 2 h during the first and last 8 h of culture time.

At the conclusion of a 24-h incubation period, flask contents were centrifuged, the resulting pellet lyophilized, weighed and assayed for DDT, PCB, nucleic acids and tritiated uridine (^3H_U) uptake.

A second experiment (Series II) identical in treatments except for uridine, was conducted to confirm stimulation patterns observed in Series I. Series II measured lyophilized harvest weights only.

A third trial (Series III) identical in design to Series I, assayed tritiated uridine uptake after five hours and eight hours incubation time to determine if assimilation rates were affected by DDT or PCB during the logrithmic growth phase. Analytical grade p,p DDT (99.9 per cent) and Aroclor 1242® (PCB—42 per cent chlorine) were used as sources of active ingredients in these experiments.

Bacteria samples were extracted with nanograde hexane and a 5 μl aliquot was injected into the gas chromatograph for analysis.

Two column systems were used: a 6 ft $\times$ $\frac{1}{4}$ in. o.d. glass column packed with 3 per cent SE-30 on 60–80 ABS chromosorb W and 2 per cent QF-1 on 60–80 ABS chromosorb W. The operating parameters were: (1) inlet temperature 235°C; (2) column temperature 200°C isothermal; (3) detector temperature 240°C; (4) flow-rate 70 cm^3 min^{-1} N_2.

Qualification was accomplished by measurement of the absolute and relative retention times of five major peaks of the PCB. Quantitation was accomplished by determination of the area under the curve of the five major peaks. Utilizing this technique, recoveries of 92.4 per cent of the PCB were experienced.

DDT and analogues were determined by direct hexane extraction of the lyophilized bacteria by gas chromotography, utilizing the identical system as described for the PCB.

RNA and DNA were measured using a modification of Agranoff's procedure (AGRANOFF and SANTEN, 1963). Radio assays utilized the Nuclear-Chicago Mark I Liquid Scintillation Spectrometer.

RESULTS AND DISCUSSION

As may be seen from FIG. 1 concentrations up to 1000 ppm of DDT or PCB impregnated into filter paper discs failed to inhibit *E. coli* growth on TSB agar.

As shown in TABLE 1 (Series I) the addition of 0.01 and 0.1 μg ml^{-1} DDT and PCB to TSB markedly stimulated *E. coli* growth above control levels. This is consistent with other microorganism work (KEIL and PRIESTER, 1969; KEIL, PRIESTER and

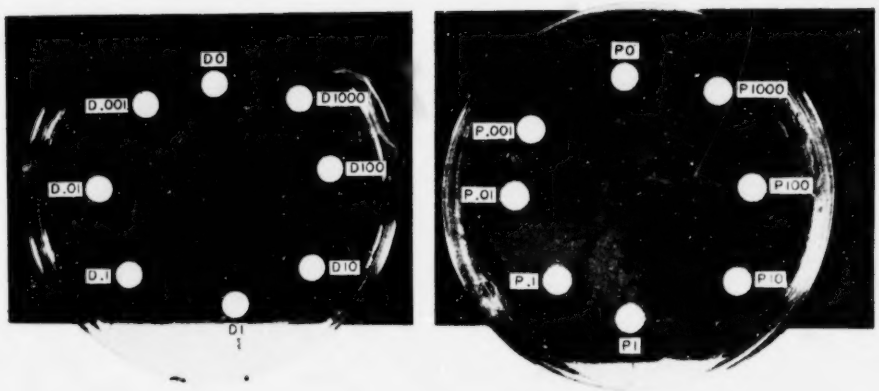

FIG. 1. Sensitivity screening of *E. coli* on TSB to 0, 0.001, 0.01, 0.1, 1, 10, 100 and 1000 ppm of A, DDT; B, PCB.

(*Facing p.* 838)

TABLE 1. LYOPHILIZED WEIGHTS AND TOXICANT UPTAKE BY *Escherichia coli* CULTURES EXPOSED TO DDT AND PCB (BACTERIAL SERIES I)

Treatment	Mean* harvest weights (mg)	Mean residue ($\mu g\ g^{-1}$)
DDT 0.1 $\mu g\ ml^{-1}$	51.1	11.2‡
DDT 0.01 $\mu g\ ml^{-1}$	115.9	2.2‡
PCB 0.1 $\mu g/ml^{-1}$	86.0	13.6
PCB 0.01 $\mu g\ ml^{-1}$	90.4	4.4
Control	44.5	0
Acetone control	27.2	1.2
Uridine control	41.6	0
LSD_{05}†	13.6	10.8

* Mean of four replicates.
† LSD_{05} = Least significant at 95 per cent probability level calculated from one way analysis of variance.
‡ Total DDT = DDT + 1.114 (DDE + DDD).

SANDIFER, 1971) showing that DDT and PCB are concentrated up to 1000 times by marine diatoms. The data also suggest that the concentration factor may be inversely related to dosage.

TABLE 2. LYOPHILIZED WEIGHTS OF *Escherichia coli* CULTURES EXPOSED TO DDT AND PCB (BACTERIAL SERIES II)

Treatment	Mean* harvest weights (mg)
DDT 0.01 $\mu g\ ml^{-1}$	126.7
DDT 0.01 $\mu g\ ml^{-1}$	176.0
PCB 0.1 $\mu g\ ml^{-1}$	186.4
PCB 0.01 $\mu g\ ml^{-1}$	153.2
Control	160.8
Acetone control	133.6
LSD_{05}†	23.6

* Mean of four replicates.
† LSD_{05} = Least significant difference at 95 per cent probability level calculated from one way analysis of variance.

TABLE 2 presents data from a duplicate experiment (Series II) but where only harvest weights were studied. This second series confirmed apparent stimulatory effects of all levels of PCB and the lowest dosage (0.01 $\mu g\ ml^{-1}$) of DDT. The highest DDT dosage did not stimulate *E. coli* growth as significantly as in experimental Series I. FIGURE 2 presents growth levels of *E. coli* in both experimental series I and II and, although culture yields differed markedly, the DDT effects parallel each other.

The results of *E. coli* residue analyses from Series I are presented in TABLE 3 and

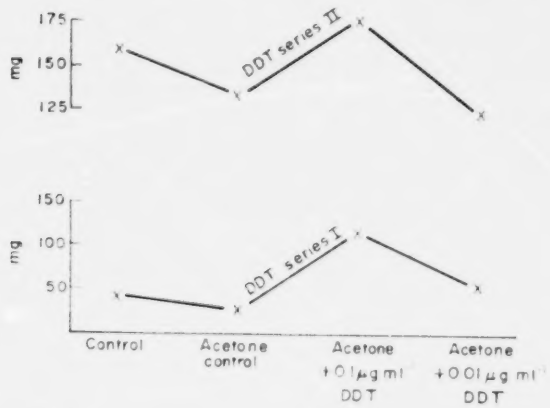

FIG. 2. *E. coli* culture yields in Experimental Series I and II showing harvest weight obtained from each treatment.

show that the organism uniformly converted DDT to DDD (75 per cent) and DDE (25 per cent). Metabolism to DDD is particularly interesting inasmuch as this step is considered to be reductive dechlorination. Aerobic and anaerobic conversion has been previously reported (MALONE, 1970; PARR, WILLIS and SMITH, 1970; WEDEMEYER, 1966), but to the authors' knowledge, this is the first indication of aerobic degradation to DDD by *E. coli*.

TABLE 3. p,p' DDT UPTAKE AND METABOLISM BY *Escherichia coli* (BACTERIAL SERIES I)

DDT in media	Residues			
	DDT	DDD	DDE	Total DDT*
	($\mu g\ g^{-1}$—mean of four replicates)			
0.1 $\mu g\ ml^{-1}$	2.1	6.0	2.3	11.2
0.01 $\mu g\ ml^{-1}$	0	1.5	0.5	2.2

* Total DDT = DDT + 1.114 (DDD + DDE).

There were no differences between treatments in DNA and RNA levels after 24 h incubation. The significance of this is not clear, since it had been anticipated that increased RNA levels would accompany growth increases. However, it is thought that differences may have occurred earlier than the 24 h time.

Data showing uridine uptake by DDT and PCB treated *E. coli* at several phases of growth are presented in TABLE 4. No significant differences in uptake between treatments and controls were evident except at 5 h incubation time, when there was a diminished ^3H_U uptake in all experimental treatments. Statistically significant diminution occurred only in PCB dosed bacteria. While not consistent with increased culture yields, the lack of change in uptake between chemically treated *E. coli* and controls does coincide with chemical assays indicating no increased levels of nucleic acids.

TABLE 4. TRITIATED URIDINE UPTAKE BY DDT AND PCB TREATED *Escherichia coli* CULTURES DURING EXPONENTIAL AND DECLINE GROWTH PHASES

Treatment	Counts min^{-1}* 5 h	8 h	24 h
DDT 0.1 μg ml^{-1}	5789	3284	2075
DDT 0.01 μg ml^{-1}	6165	2845	2391
PCB 0.1 μg ml^{-1}	4342	2250	2192
PCB 0.01 μg ml^{-1}	4756	2367	2462
Uridine control	8020	2675	2389
LSD_{05}†	2800	979	859

* Mean of four replicates.
† Least significant difference at 95 per cent probability level calculated from one way analysis of variance.

Results from these experiments indicate that DDT and PCB stimulate *in vitro* growth of *E. coli*. If this phenomena occurs *in vivo* further concern is justified since any environmental contaminant which affects indicator organisms may result in a distorted portrayal of an actual situation.

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TOXICOLOGICAL PROBLEMS ASSOCIATED WITH USE OF PESTICIDES

Dr. Wayland J. Hayes, Jr.

Chief, Toxicology Section, Technology Branch, Communicable Disease Center,
Public Health Service, U. S. Department of Health, Education, and Welfare

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TOXICOLOGICAL PROBLEMS ASSOCIATED WITH USE OF PESTICIDES

Dr. Wayland J. Hayes, Jr.

Chief, Toxicology Section, Technology Branch, Communicable Disease Center,

Public Health Service, U. S. Department of Health, Education, and Welfare

Abstract

BECAUSE the basic pharmacology of pesticides, the potential health problems of residues, and the practical problems of occupational health are so interrelated, and because the number of trained men available to investigate the problems is so limited even in the most advanced countries, progress on the toxicology of pesticides will be made most rapidly if there is good communication among all scientists and regulatory officials in the field.

Poisoning is known to have been caused by at least 49 pesticidal chemicals. The clinical effects may be diverse and complex. With minor exceptions, the safety record of pesticides in North America and Europe is good. For example, the death rate from this cause in the United States is 1.0 per million population. The record in some other countries is poor and it may be even worse in places where experience in the use of chemicals is small and the collection of vital statistics is fragmentary. There may be situations in which countries should prohibit the manufacture or importation of specified pesticides.

The conditions of use are at least as important as inherent toxicity in determining the safety of a pesticide. Useful precautions have been recommended, but more information is needed on exposure to, and absorption of, pesticides under different conditions.

Measures to promote safety include technology, three kinds of laws, education, clinical tests to permit limitation of exposure, and special protective devices. Safety regulations could be improved by requiring the publication of data justifying all permitted uses and by demanding greater continuing attention to the health of people with maximal exposure to pesticides.

Introduction

The great increase in the production of pesticides in the United States since about 1945 is shown in Figure 1. Production has increased in many other countries. Because of imports, the use of pesticides is somewhat more general than is the manufacture of these compounds.

Not only have the number of pesticides and their tonnage increased, but these materials are now employed for a wider range of uses and applied in ways that were rare or unknown only a decade or two ago. For example, in California the area of cropland treated with pesticides by aircraft

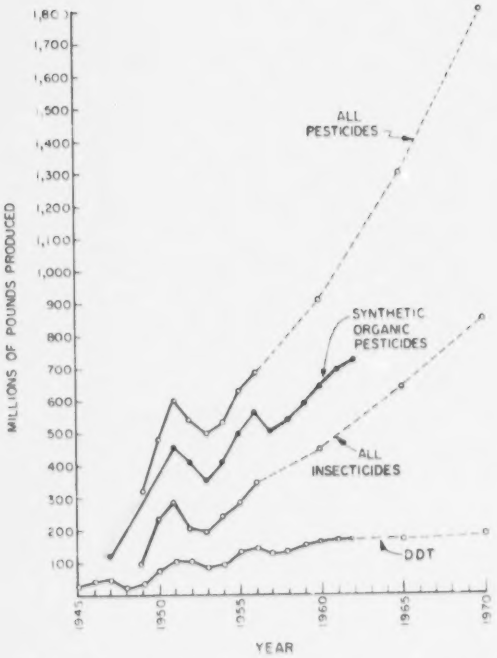

Fig. 1. United States production (solid lines) and predicted production (dotted lines) of DDT, all insecticides, synthetic organic pesticides, and all pesticides. (Data from Commodity Stabilization Service, U. S. D. A. and estimates from Fisher, 1956 and President's Materials Policy Commission, 1952.)

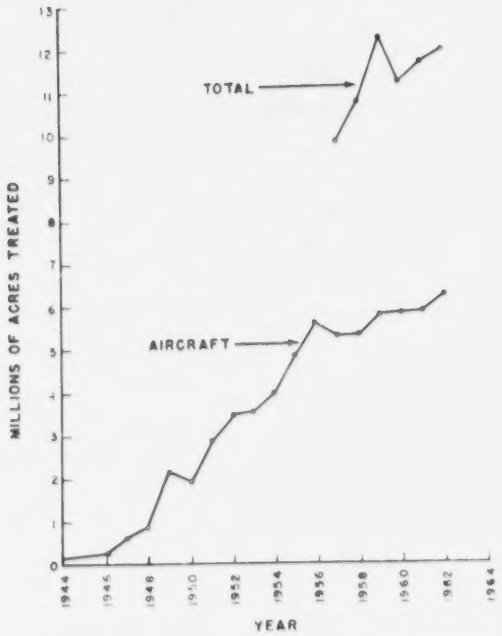

Fig. 2. Area of land in California treated with pesticides in successive years by means of aircraft and by all methods combined. Records for 1934 to 1944 show that the largest area treated by aircraft in any year during that period was 223,074 acres. (Based on Annual Reports, California Department of Agriculture and on Announcements of the California Bureau of Chemistry.)

increased from 132,701 acres in 1942 to 6,272,880 acres in 1962 (Fig. 2).

All of these factors tend to produce potential, if not real, hazards. The basic pharmacology of pesticides, the potential health problems of residues, and the practical problems of occupational health are interrelated, and the number of trained men available to investigate the problems is limited even in the most advanced countries. Under the circumstances, it is difficult to overemphasize the need for communication among all scientists and regulatory officials interested in one or more aspects of the problem.

This paper is intended as an outline only. It does serve to draw attention to reviews and reports giving essential details and original references regarding several of the topics under discussion.

Hazards Associated with Pesticides

Nature of Poisoning

Poisoning of people has been caused by at least 49 pesticidal chemicals—including representatives of all the major classes. The list would be longer if the inorganic compounds of each element—for example, arsenic—were counted separately. Descriptions of some of the more important kinds of poisoning are given in the "Clinical Handbook on Economic Poisons".[31] In most instances, human illness is clinically similar to the disorder that can be produced easily in animals, and it occurs rather promptly after excessive exposure. No organ system is immune. Many pesticides have multiple effects.

There are exceptions to the rule that poison-

ing in man resembles that in animals and is prompt in onset. Injuries such as hay fever, asthma, and anaphylaxis, sometimes caused in humans by pyrethrum, can be produced in animals only with difficulty, if at all. Myelogenic blood dyscrasias involve some aspects of allergic reaction, and cannot be studied usefully in animals. Chlordane, benzene hexachloride, and DDT have been credited with causing such blood dyscrasias in man in very rare instances. Some other pesticides cause disease in man but in few laboratory animals. Thus, mipafox (once proposed as a pesticide) and the much better known triorthocresylphosphate (TOCP) produce their typical paralytic effect only in people and chickens.

A few compounds produce illness that may be delayed for days or weeks after exposure. For example, paralysis occurs 10 to 14 days following ingestion of TOCP at a time when the compound and its metabolites have already been excreted. The very different injury to the nervous system produced by the alkylmercury fungicides may become apparent during a period of exposure, but in other cases may not be clinically evident for as much as several months after last contact with one of these poisons. Another fungicide, hexachlorobenzene, is peculiar because appearance of its toxic effects depends, in part, on exposure to light.

When biologically active chemicals are absorbed at or about the same time, their effects may be additive or they may tend to cancel or to potentiate one another. Potentiation between the members of certain pairs of organic phosphorus compounds is well known. Murphy and Dubois [45] showed that the potentiation of EPN by malathion depends on competition for an enzyme site, but pointed out that compounds that react in this way need not be of the same chemical class. In fact, a slight potentiation of parathion by a tranquilizer, promazine, was demonstrated in rats [26] and suspected in man.[3]

Illness produced by pesticides may be acute or chronic. These terms are frequently not understood by the laity and are often misused in the literature on economic poisons. The common mistake is to suppose that the terms, "acute" and "chronic," have something to do with the severity of illness or with the duration of the exposure responsible for poisoning. Acute disease is any illness of relatively brief duration such as parathion poisoning—or a cold or plague. Chronic disease is illness that lasts for a long while so that there is tissue change, often characterized by scarring. Examples are the neurotoxic effects of mipafox and related compounds—or dandruff or leprosy. Some compounds, such as parathion, that produce acute poisoning are not known to produce chronic poisoning. Parathion poisoning ranges from extremely mild to fatal. It may be produced by a single dose or by repeated exposure. Some materials, such as lead, may produce either acute or chronic poisoning, depending on conditions; a single dose may produce an acute phase of poisoning followed by a chronic phase.

All compounds that are absorbed may be said to be "stored" as long as they remain in the body. The degree and duration of storage depends on the compound, on the intensity and duration of dosage, and, to a lesser degree, on other factors. The storage of DDT and its metabolite DDE has reached a steady state in people in the United States.[32] Dieldrin was recently demonstrated in people of England,[35] and benzene hexachloride was measured in the people of France.[33] Both dieldrin and BHC occur in the general population of the United States also.[13]

Although most of the major effects of pesticides and some of their minor effects are known, there can be no assurance that the picture is really complete. On the other hand, the fact that knowledge is never complete is exploited by a few food faddists [52] and others in ways that are distinctly harmful. Occasionally a case is published as an example of poisoning in violation of clinical and epidemiological evidence.

What has been said should suffice to show that the clinical effects of poisoning by pesticides may be diverse and complex. An isolated case may occasionally tax the diagnostic ability of a physician, even one specialized in toxicology. On the other hand, if dangerous compounds are involved, and if the hygiene of workers is sufficiently poor, cases will begin to appear in a pattern that even

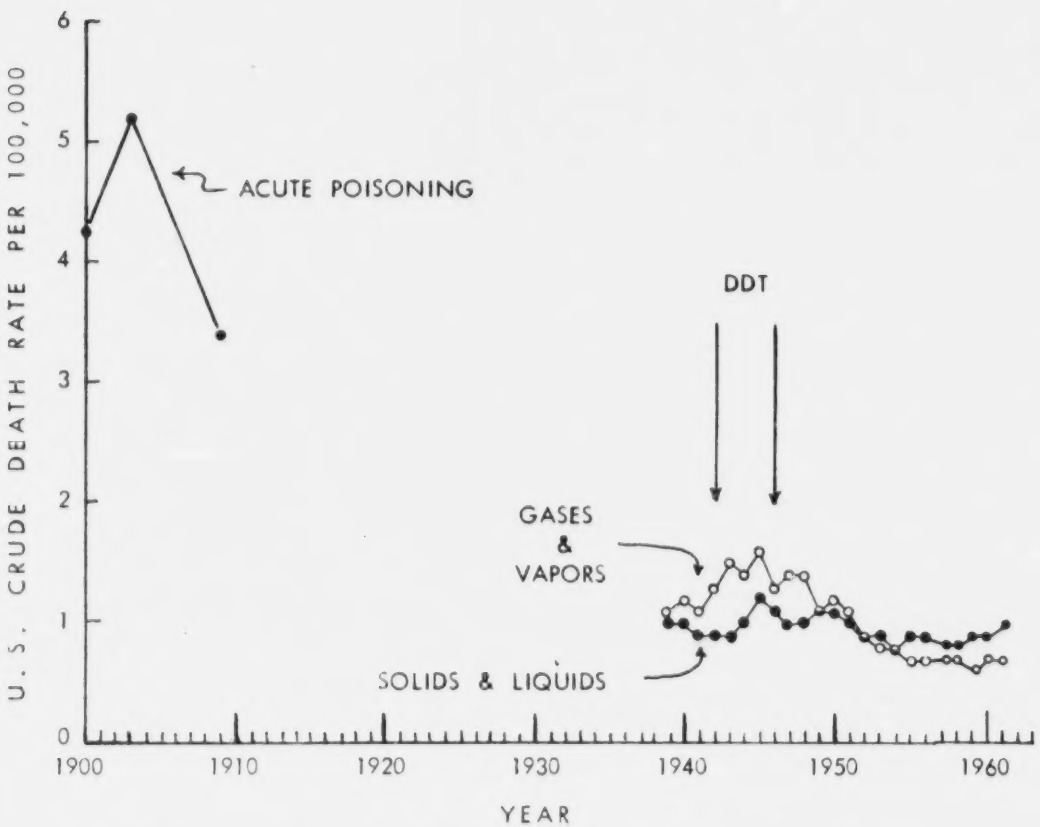

Fig. 3. Mortality in the United States associated with accidental poisoning. (Based on Vital Statistics of the United States.)

the layman can recognize. For example, in the episodes of dieldrin poisoning among malaria control teams of several countries,[29] the problem was recognized by the laborers themselves.

Frequency of Poisoning and Underlying Circumstances

Separate reports have indicated a ratio of nonfatal to fatal cases ranging from 1:13 to 1:750. In the first instance, only severe cases were considered; in the second instance, extremely mild or even questionable cases were counted. A ratio of 1:100 is as nearly accurate as may be stated at this time. Even in cases of mild poisoning by pesticides, the exposure of the patient has been substantial. The dosage in more severe cases has been proportionally greater.

In the United States, annually from 1939 through 1961, there were between 1,191, and 1,804 deaths from all solid and liquid poisons. (Fig. 3). Detailed analysis showed that from 1946 to 1956 pesticides were responsible for 7.8 to 12.8% of these deaths, or about 150 per year. Although fumigants did cause a few deaths, they constituted only about 0.7% of the deaths caused by gases and vapors. The death rate associated with all pesticides in the United States is 1.0 per million population.

Each year in the United States, the majority of deaths caused by pesticides has involved children who found and ingested one of the compounds. The children obtain the material from bottles or packages left within their reach. Unlabeled containers, especially soft drink bottles that may be used in mixing, constitute a particular hazard. Many children have been killed by poison remaining in packages that had been discarded as empty. Another factor important in some cases is the greater inherent susceptibility of children to some poisons, notably parathion.

It was somewhat unexpected that at least as late as 1956, the last year for which complete information is available, insecticides older than DDT were still responsible for the majority of deaths.[30]

The safety record of pesticides in the United Kingdom is good also,[18] and with minor exceptions, the same seems to be true of Europe generally.

The statistics for Japan are in marked contrast to those for the United States and Europe. According to Namba,[46] there were over 3,000 deaths from parathion in Japan during the 6-year period 1953 through 1958. Note that this figure is incomplete—that it includes parathion only. It seems likely that new pesticides caused more trouble than old ones and that among accidental cases the occupational ones predominated in Japan. Even if one considers only those cases of poisoning by parathion that do not involve suicide, not all reasons for the marked contrast in statistics for the two countries are clear. In Japan the total amount of pesticides used is probably less per capita than in the United States, and there is no evidence of more intensive application to any particular crop than for certain crops in the United States. One explanation is the relative importance of hand application in Japan. There is another difference for the two countries that seems worth mentioning although it is not related to toxicology, i.e., the character of the need to use pesticides. Namba[46] states: ". . . since the introduction of parathion in rice cultivation, the rice crop has soared, and the problem of shortage of food has been solved."

In yet another country the death rate from pesticides is more than twice that in the United States, while the use of organic insecticides is only one-sixteenth as great (Table 1).

Table 1

RELATION OF INSECTICIDE USE TO MORTALITY FROM INSECTICIDE POISONING

Annual Rate	*U.S.A.*	*R.V.*
Use of Insecticides (grams per capita)	600	36
Mortality (deaths per million)	1	2

There are vast areas of the world where pesticides are used but where vital statistics are collected only in a very fragmentary way or not at all. In general, it is in these same areas that experience in the use of chemicals is least and the ability to read and understand labels is infrequent. A few reports of poisoning have emerged from such places. Some of these reports specifically state that they are incomplete and do not cover all the area in which poisoning probably occurred. We even have the curious information that modern pesticides have been used by a witch doctor as test poisons.[34] However, it seems most unlikely that there is any real information on the amount of injury already done by the agricultural use of pesticides in some parts of the world. There are good reasons to think that more extensive use of pesticides in some developing areas would cause significant injury. In anticipation of this and related problems, a joint meeting of the WHO Expert Committee on Pesticide Residues and the FAO Panel of Experts on the Use of Pesticides in Agriculture[63] concluded that there might be situations in which some countries would prohibit the manufacture or importation of specified pesticides.

Much of what has just been said regarding differences between advanced and developing countries applies also to illness and death resulting from foods and other commodities becoming contaminated with pesticides during transport. Although accidental contaminations of food[11] and clothing[58] have occurred in technically advanced

countries, the most severe instances reported were in developing countries. For example, more than 100 deaths occurred throughout the State of Kerala, India, as a result of the distribution of grain contaminated by parathion.[38] Other outbreaks and the entire problem of residues have been reviewed by Durham.[15]

Occasional accidents due to contamination of food with pesticides during transport undoubtedly will continue to occur despite reasonable precautions. However, experience has shown that in such unfortunate episodes quick, decisive action can hold injuries to a minimum. For example, at Singapore an announcement was made by the Ministry of Health only 16 hours after the first case was admitted to a hospital in what proved to be an outbreak of poisoning by parathion-contaminated barley. Radio programs were interrupted every 15 minutes to broadcast a warning, and vans equipped with loudspeakers toured the streets spreading the news.[37]

The few reports of food becoming contaminated through grossly improper application of pesticides have come from the United States.[41, 44] Fortunately, no deaths have resulted. This sort of mistake is more likely to occur, but less likely to be reported, in countries where the average level of technical training is lower.

Somewhat related is poisoning by seed-grain intentionally treated with pesticides and never meant to be eaten. In Turkey over a period of years, thousands of cases of poisoning and many deaths resulted when people ate grain treated with the fungicide hexachlorobenzene.[9, 51]

In a freak accident, a Korean family of 14 persons lived for 15 days on a diet consisting almost entirely of corn meal containing warfarin intended for military use in rodent control. Twelve of the family survived a dosage of warfarin that would have decimated a population of rats.[39]

The preceding paragraphs dealt with the overall statistics of poisoning and with some details of the major causes of poisoning. The three main sources of recorded fatal cases are (a) individual accidents involving ingestion or other gross exposure of children and others, (b) occupational poisoning of applicators, and (c) group accidents resulting from eating contaminated food. Minor and potential hazards may now be mentioned.

Occupational poisoning in the manufacture of pesticides has been infrequent and has resulted in very few deaths. This is true despite the high concentrations of pesticides and the frequent presence of toxic precursors involved in synthesis. This good record is made possible through knowledge, discipline, and good plant design. It is the relative absence of these factors that makes agricultural application of pesticides more dangerous than their manufacture. Formulators seem to occupy a position midway between manufacturers and applicators. The function of formulators is to dilute pesticides with solvents, dusts, or other carriers, so these men have potential exposure to concentrates and to large volumes of dilute preparations. Measurement of storage or excretion of pesticides in these workers shows that they have a high, continuing level of exposure. In spite of this, they usually escape the occasional massive exposures that prove the undoing of many applicators.

So far there have been no reports of poisoning of transport workers resulting from exposure to pesticides, although the contamination of food by pesticides during shipment has led to many deaths and the contamination of clothing in transport has led to serious illness. The reason for the escape of the transport workers is undoubtedly the brevity and relative remoteness of their contact.

A curious form of occupational poisoning is the intoxication of crop workers by residues of parathion.[30] Episodes of this kind of poisoning have been distinguished by the high incidence of cases in the small number of groups affected; by relatively mild, uniform symptomatology; by occurrence almost exclusively in connection with tall crops; and apparently by a far greater prevalence in some years than in others.

There have been no cases of poisoning by pesticides on food following their application according to label directions.

The possibility that mild poisoning may be a contributing cause of accidents cannot be ruled out with any such certainty, but the increased application of pesticides by aircraft has not been accompanied by an increase in the rate of aircraft accidents (Table 2).

Table 2

AIRCRAFT ACCIDENTS IN AERIAL APPLICATION ACTIVITIES*

Year	Accident Rate per 100,000 Hours	
	Total Accidents	Fatal Accidents
1951	51.68	7.91
1952	53.16	6.92
1953	47.92	6.37
1954	46.16	6.34
1955	37.21	6.34
1956	34.51	5.61
1957	42.73	4.96
1958	39.67	5.84
1959	35.69	6.47

* Data provided by the Civil Aeronautics Board.

Importance of the Conditions of Use in Determining the Safety of Pesticides

The conditions of use are at least as important as inherent toxicity in determining the safety of a pesticide. One of the variables is formulation, and its importance is illustrated by Table 3 copied from Goldblatt.[27] This table involves the concepts of formulation and route of exposure and implies two other variables: compound and dosage. What does this mean? Is there any flexibility, or are all the so-called "variables" really fixed entities in any given control situation? Are there any other variables that need to be considered?

It is true that in order to achieve control in many situations the compound, formulation, and application rate are limited or even fixed by the susceptibility of the pest, the cost of different compounds, and other practical considerations. Of course, if one really can select between a safe and a dangerous compound, the choice is easy. But there are other variables that determine how much dosage the applicator will get when all other conditions remain constant. These variables may be summarized under the heading, "Technique of Application," and include:

1. Equipment for application (and its state of repair).
2. Protective devices.
3. Personal hygiene.

Many of the precautions prescribed by the World Health Organization[62] for spray operators on public health teams apply with equal force to agricultural workers.

Clearly the conditions of exposure of a pilot are very different from those of a man operating a ground machine, even though both apply the same formulation at the same rate per hectare. The difference is even greater if one considers the old spray machines pulled by a draft animal and the new ones pulled by a tractor. With the first, the worker walked directly behind the spray nozzles; with the second, he sits high and dry above and ahead of the nozzles. The record shows what one would expect; i.e., a relatively high proportion of workers have been killed as a result of using old-fashioned sprayers. And yet, the use of sprayers pulled by draft animals may be necessary, especially in developing countries. It has been claimed that the safety of tractors could be improved by a little attention to design. Would it not be even simpler to change the design of animal-drawn sprayers so that the worker goes in front of the nozzles rather than following, drenched, behind them?

Sometimes the objection is heard that workers will not, or even that they cannot, tolerate protective devices. Experience shows that when they understand the problem and have adequate supervision they *will* use clothing that offers more protection than what they ordinarily wear.[29] If hot weather and other conditions make a respirator or mask impractical, then a veil will offer a remarkable degree of protection to the skin of the face and neck and to the respiratory tract.[60] Additional protection can be realized by limiting the number of daily work hours and, of course, through good personal hygiene.

One really ought to count the provision of water and soap as a protective device, for it is only when they are ready when needed that an inclination to good personal hygiene can become

Table 3

RELATION OF THE PHYSICAL STATE AND DISPERSION OF A COMPOUND TO THE LIKELIHOOD THAT IT WILL PRODUCE POISONING IN PEOPLE EXPOSED BY DIFFERENT ROUTES.

State	Route			Remarks
	Lungs	Skin	By Mouth	
SOLID:				
Lumps	—	+	±	Unless material is deliberately or accidentally taken by mouth, absorption by this route is unusual in practice; can occur from eating or smoking during work.
Large particles	+	+	±	
Small particles (dust)	+++	+	±	
FUME, VAPOUR AND SMOKE:	+++	±	±	Ditto
LIQUID:				
Native	±	+++	±	Ditto
SPRAY:				
Emulsion of solid or liquid	++	+++	±	Certain solvents may enhance skin absorption.
Solution of solid	++	+++	±	
Dispersion of solid	++	++	±	
GAS:	++++	±	±	Some gases can pass through the skin.

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effective. Devices and hygiene are related in other ways, and the relation may be negative. For example, it may be more dangerous to wear contaminated gloves than to wear no gloves at all.

Eventually, one must make a decision about what protective equipment to use. Table 3 gives some valuable hints but gives no indication of quantitative requirements. The techniques for measuring exposure to and absorption of pesticides have been reviewed in two papers by Durham and Wolfe,[16, 17] who also recorded the results obtained so far with these techniques in various parts of the world. In spite of a good beginning, many more measurements need to be made in order to evaluate the relative importance of different protective devices in different situations. Table 3 strongly suggests that dusts as well as vapors constitute a special respiratory hazard, but as already observed, the quantity is important.

Measures to Promote Safety

Technology

As suggested in the previous section, all protective measures must be based on knowledge in order to be valid. This knowledge depends, in turn, on an adequate technology. Frequently, information obtained about a compound in one country can be used without modification in another country. This is most likely to be true in relation to the public health aspects of the problem (as compared to the agricultural aspects), because man's environment—especially his internal environment—is relatively stable. By contrast, there may be great variation in the temperature, rain, wind, and radiation to which crops are exposed, and all these uncontrollable factors are important in determining the rate at which any given deposit of a pesticide is lost from a crop. Other variables include the kind of chemical, the kind of formulation, the kind of plant, the number of applications, and the schedule of application.

It requires the careful cooperation of agricultural specialists and chemists to determine what residues of pesticide will be present at harvest following a given scheme of treatment. Of course, such studies are worthwhile only after it has been established that the particular chemical is useful in controlling the pest for which it is intended.

It is meaningless to consider the effect of any chemical on health except in terms of dosage. There is some variation in the requirements for study of pesticides in different countries. However, all agree on the most important points, as follows:

1. Provision of an analytical method.
2. Determination of routes of absorption.
3. Establishment of dosage levels that produce insignificant injury (by different routes).

4. Exploration of characteristic pharmacologic effects, including measurement of storage.

All agree it is desirable to determine the basic mode of action, but this has been done in only a few instances. A number of booklets[10, 40, 47, 48, 61] have been prepared to guide toxicological studies. Barnes and Denz[6] have reviewed the scientific basis of such studies.

In general, governments require manufacturers of pesticides to provide the agricultural and toxicological information necessary to determine the safety of each use of each pesticide both to the crop, and to man and useful animals. However, it is important that the government have enough staff with sufficient training to evaluate the results presented by industry. Furthermore, when necessary, the government must be capable of checking the technical accuracy of information provided by industry, either through its own laboratories or through cooperation with other governments.

Unfortunately, a portion of the information on toxicology obtained to fulfill legal requirements regulating pesticides is not published. Publication of evidence for safety should be required by the same laws that require labeling and tolerances and thus permit sale. This legal device would reduce the cost of regulating pesticides for smaller nations whether they are technically advanced or not. This is true (even though the manufacturer is required to supply information on his own product) because there is a hidden cost in assembling information on other compounds and in assembling the views of toxicologists who have done research either on the same or related compounds. Obviously, regulatory decisions should be based on all existing, relevant information. If even a single country required the publication of data, this would go a long way toward correcting the present deficiency. There would be no disadvantage to the manufacturer. He would have little or no increase of cost. There certainly could be no disadvantage to publication of data showing that a product is safe for its intended use.

The laws discussed in the following section require that the safety of each compound be established for every use specified. The toxicological information required under these laws is based alalmost exclusively on animal studies. Of course, animal studies are basic, but more attention should be paid to man himself. First, careful studies should be made of formulators, applicators, and others with maximal intensity and duration of exposure. The reasons for such action were stated for drugs by Macgregor and Perry.[42] The same reasons apply to pesticides. Briefly, (a) not all hazards are known; (b) of those that are known, not all can be adequately studied in animals; and (c) it is only by organized accumulation of information on toxicity to man that "control measures may be based on knowledge and not on ignorant guesswork. . . ." It is chiefly in this careful observation of men with intensive and prolonged exposure that investigators in developing countries have made important observations and can make even more important contributions in the future.

Second, certain information can be obtained only through studies on volunteers. To be sure, dosages for volunteers are generally restricted to lower levels than those that actually occur in use, and there are practical limitations on the duration of such studies. However, work with volunteers makes it possible to study the relation of absorption, storage, excretion, and physiological effect to dosage. Not only may each of these parameters be different in man as compared with experimental animals, but some physiological functions, such as reaction time, visual acuity, and ability to judge distance, may be studied in animals only with so much difficulty that the results are hard to interpret even for the species under study.

Laws—There are three main kinds of regulations for pesticides irrespective of the basic legal structure:

1. Regulation of labeling under a system of registration.
2. Regulation of residues under a system of tolerances.
3. Direct regulation of use.

In practice, different countries use regulation of residues or regulation of use, or both, in addition to regulation of labeling.

An examination of the record of pesticides in different countries shows that proper labeling is the most important factor in determining safety. Poor labeling has resulted in many accidents even in countries with highly developed technology and essentially universal literacy. The way in which good labeling is achieved is of secondary importance only. In the United States of America, good labeling is required by very detailed regulations under the Federal Insecticide, Fungicide, and Rodenticide Act.[55, 56, 57] In Great Britain, good labeling is obtained just as surely through a cooperative arrangement between Government and the manufacturers of pesticides. The chief information that should be contained in a good label is as follows:

1. Name and address of the manufacturer.
2. Name of the product.
3. Net content of container.
4. Name and concentration (by weight) of each active ingredient and the concentration of the inactive ingredients. The manufacturer should be required to submit for the record of the Ministry of Agriculture a complete statement of the composition, including the name and concentration of each inert ingredient.
5. Adequate directions for the control of each pest for which the manufacturer claims control. The directions should include the rate of dilution, the rate of application, the time of application and, in the case of food crops, the limitations necessary to avoid excessive residues. Limitations may include interval between application and harvest in terms of days or the development of the plant (e.g., "when fruit begins to form").
6. Adequate precautionary labeling to prevent injury to persons applying the product or to persons, animals, or plants coming in contact with the product. Highly toxic products should bear the skull and crossbones, the word *"Poison,"* and information on treatment of intoxication. In order to provide a clear distinction between very hazardous materials and those of lesser hazard, the skull and crossbones and the word *"Poison"* should not be used indiscriminately.
7. Permit number of the Ministry of Agriculture.

It is of the utmost importance that these items be clearly and prominently displayed on the label and that the entire label be printed in the language of the country in which the product is to be used. This means, among other things, that all the basic information must be shown on a label attached to each container; separate leaflets may supplement but not replace the basic label. Further protection is offered if the regulations permit control of packaging, including the size of containers offered for sale. Another desirable feature is control of advertising, which is only an extension of the label claims of a product. Enforcement is promoted if regulatory authorities are authorized to inspect the facilities and records of manufacturers, formulators, shippers, and distributors of pesticides. Shipments of pesticides must be sampled at retail stores to determine compliance with the law. Review for compliance should be done by the same laboratory and administrative personnel as those responsible for initial registration. Good administration of regulations of labeling requires the cooperation of people with different training, including entomologists, agronomists, chemists, toxicologists, and attorneys. Of course, the law must provide suitable punishments for violations.

Illiteracy poses a tremendous obstacle to realizing the benefits of good labeling. However, this is no reason to underestimate the importance of labeling, for even if farm workers cannot read, good labeling is the best single way of getting information on pesticides to them through the farm owner or foreman.

Any effective regulation of residues of a pesticide depends on:

1. Determination of effective dosage and schedule of application.
2. Measurements of residues that result from this anticipated use under the local climatic and other environmental conditions involved with each separate crop.
3. Determination of a dosage of the pesticide that produces no significant change in experimental

animals even when given for long periods of time.

4. Establishment of a tolerance that
   (a) offers a suitable margin of safety (usually at least 100) as compared with dosage determined under No. 3, but
   (b) is in no case significantly greater than the residue that will result from proper use of the particular pesticide on the crop in question.
5. Analysis of residues of the pesticide on the crop as offered for sale.
6. Punishment for violations.

It must be understood that all of these factors must be taken into account whether chief dependence is placed on regulation of residues or on regulation of use. In fact, the work involved in the first four factors is identical under both systems. The differences, involving factors 5 and 6, are relative. If emphasis is placed on regulation of residues, many highly trained chemists (in addition to those necessary under factor 2) are required for effective police action. If emphasis is placed on regulation of use, excessive residues will be prevented to a large extent through supervision and education of farmers following visual inspection of their procedures. Such sampling of residues as may be necessary may be done far more efficiently, because the inspectors know from observation exactly what produce is likely to bear excessive residues. Inspectors may have some knowledge of the origin of produce at shipping points or on the open market.

Even when emphasis is placed on the regulation of residues, some selection of samples for analysis must be made; enforcement of such a regulation attempts to minimize violations, not to determine complete statistics on the concentration of each pesticide in the average diet. No country has attempted to divert enough chemists and laboratory equipment to accomplish continued, unselected or random sampling of the total diet. The distinguished British toxicologist, Barnes,[5] warned against the danger of diverting the efforts of too many skilled technicians from productive to regulatory work.

There is considerable advantage in having labeling regulated by the Ministry of Agriculture and residues regulated by the Ministry of Health. Whether this is done or not, it is important that the activities of the two ministries be closely correlated in this regard. Competence in both agriculture and health must be involved no matter what the regulatory pattern.

The direct regulation of use of pesticides is most fully developed in Great Britain.[28] There is no Federal law of this kind in the United States of America but several of the states, notably California[8] and Florida,[24a] have laws regulating use. Such laws may not have all of the following features, but the first feature is basic:

1. Establish a list of dangerous materials subject to regulation. Compounds may be listed because of danger to human health, other crops, or both. Compounds that have been listed are: various arsenicals, parathion, methyl parathion, tetraethylpyrophosphate (TEPP), EPN, Phosdrin, Guthion, demeton (Systox), schradan (OMPA), Chipman 6199 (Tetram), phorate (Thimet), Di-Syston, endrin, aldrin, dieldrin, Telodrin, and 2,4-D and related compounds (danger to crops only).
2. Designate a regulatory authority.

3a. List instructions and protective equipment that must be supplied by any employer involved in the use of compounds listed under No. 1. This list may include approved masks, gloves, and clothing, as well as specific instructions for use.

3b. List obligations of any employee engaged in the use of these compounds. Briefly, the employee must follow instructions and use the equipment provided.

4. Require that anyone buying a dangerous material must obtain a permit from the regulatory authority for each use, stating the time, place, and crop involved.
5. Forbid any dealer to sell a dangerous material to anyone except on presentation of an official permit.
6. Require that employers engage a physician to supervise and carry out prescribed tests on em-

ployees who will be exposed to specified dangerous materials.

7. Provide a schedule of fines or other punishments for employers, employees, or dealers who violate any provision of the regulations.

In addition, several states have special laws for licensing of pest control applicators, pilots, and others with maximal exposure to pesticides.[24b]

The official who issues permits should be equally familiar with good agricultural practice and with the hazards of misuse. The authority is commonly vested in a county or district commissioner of agriculture. Much of the work of inspection and enforcement is educational in orientation and closely related to agricultural extension work. On the other hand, it is important that the regulation carry firm sanctions so that willful or repeated violations may be punished. It is my judgment that it is more efficient to prevent misuse of pesticides through persuasive education of farm owners and farm workers than it is to detect excessive residues and take appropriate legal action after food has reached the open market.

Regulation of use of pesticides contributes simultaneously to occupational health and the control of residues.

Regulation of use is closely related to occupational health, but in connection with pesticides the former has been associated traditionally with Ministries of Agriculture while the latter has been associated with Ministries of Health. Regulation of use involves prevention of excessive residues; protection of crops from herbicides; and other functions, quite distinct from any consideration of hazards to agricultural applicators. This latter function is really occupational health for farmers. However, public relations are improved and expense is reduced by having farmers contacted by extension agents and manufacturing and formulating workers contacted by experts in occupational health.

Occupational health has both engineering and medical aspects. The former includes ventilation, factory design, and application of threshold limit values[1] for the concentration of chemicals in the air of workrooms. The latter includes physical and laboratory examination of exposed workers. In some instances, biological threshold limits (levels of the chemical or its metabolite in blood or urine) are more valuable than threshold limits for air.

Education

Education plays a far more important role in the safe use of chemicals than is frequently realized. The simplest and most generally useful message is: "Read the label." It cannot be emphasized too often that a good labeling program is the most important factor in safe use of pesticides. People need to be educated to use the information on the label, which is the product of so much study and care.

Teaching devices include informal discussions, lectures, movies, filmstrips, small leaflets, posters, and pocket cards.

The Ministry of Health has a special responsibility to train physicians in diagnosis and treatment. The Ministry of Agriculture has a special responsibility to farmers. Extension programs offer an unexcelled opportunity to bring accurate information to applicators irrespective of their state of literacy. Pesticide manufacturers, especially those that produce highly toxic compounds, have a responsibility to train people who work with these materials. One large manufacturer of parathion has spent thousands of dollars in the United States of America studying and reporting cases of poisoning, developing medical tests for subclinical effects, and distributing leaflets, posters, and cards to point out the hazard of the compound and the precautions necessary in its use. The relatively good safety record of parathion in that country is due largely to this educational effort.

Tests to Permit Limitation of Exposure

Tests of exposure and absorption were mentioned above in connection with the problem of evaluating the importance of different routes of absorption in order to guide the selection of protective devices. Tests of absorption as measured by excretion may also make it possible to compare the degree of effective exposure of different groups and different individuals, irrespective of route of

exposure. Significantly greater excretion in a group or in any individual of a group indicates a need for improvement in one or more aspects of hygiene. Careful medical examination may eventually show that a particular level of excretion is associated with mild poisoning in some people. A biological threshold limit is then set at a lower level to offer a margin of safety. An important benefit of such tests is that they keep workers reminded that the absorption is real and measurable. Their usual response is to handle the poison more carefully. It may be helpful to start a competition to see who can show the lowest absorption in spite of satisfactory production.

Urinary excretion of the following pesticides can now be measured in workers: lead,[21, 36] arsenic,[59] mercury,[54] parathion and other *p*-nitrophenols as *p*-nitrophenol,[20] malathion as phosphates,[43] dinitro-*o*-cresol,[49] carbaryl as 1-naphthol,[7] pentachlorophenol,[22] DDT as DDA,[12], and dieldrin.[11] Unpublished research from our laboratory indicates that the urinary excretion of *p*-dichlorobenzene as 2,5-dichlorophenol may be measured in man as was shown earlier in rabbits.[4]

Most chlorinated insecticides are eliminated at least in part in the urine and presumably could be measured as organic chloride. This method has been specifically suggested for detecting chlordane[53] and toxaphene,[2] but apparently it has not been used for monitoring people. In a similar way, the derivatives of organic phosphorus compounds could be measured as phosphorus, but so far the method has been used under practical conditions for malathion only. The relatively nonspecific methods for detecting chlorine and some other elements are being replaced rapidly. Gas chromatography followed by microcoulometric detection of chlorine—already used for dieldrin and its metabolites—offers a far more dependable method for chlorinated compounds. The same system with electrodes sensitive to sulfur may be used for many organic phosphorus compounds.

Some compounds, for example dinitro-*o*-cresol,[49] can be measured in the blood of workers.

Although it is a common practice to take blood samples in order to measure the concentration of materials "stored" in the blood, the sampling of other tissues has practical limitations. However, only a minor biopsy is needed to sample fat; and some compounds, including the chlorinated hydrocarbon insecticides, are preferentially stored in this tissue. The concentration of a pesticide in fat may reach 50 to 100 times that in other tissues and tends to correlate with the concentration of neutral fat in the tissues. Utilization of this difference leads to ease of analysis and gives the highest possible sensitivity in relation to any given level of absorption.

Measurements of physiological effects may be used in much the same way as measurements of pesticides or their metabolites in blood or urine. Thus, the measurement of blood cholinesterase activity gives some indication of the absorption of organic phosphorus insecticides. Although the test is of some value in following individual clinical cases, it cannot be used to determine or predict clinical state. An important reason is that the body adapts to high levels of acetylcholine so that the rate of depression of cholinesterase, as well as the degree of depression of this enzyme, must be taken into account. Thus, a sudden slight depression is often associated with moderate illness, while a very gradual severe depression is compatible with good health. In almost all instances, severe poisoning by an organic phosphorus insecticide is accompanied by a severe depression of blood cholinesterase. A few cases indicate that even this relationship is not universal, but an explanation for the exception is not known. In spite of the complexity of its interpretation, the cholinesterase test is valuable as a measure of occupational hygiene.

In reviewing this matter, the Expert Committee on Pesticides[62] suggested that spray operators be withdrawn from exposure if the activity of their blood cholinesterase decreases by 25 percent or more from a well-established pre-exposure value. Others have suggested 50 percent as the degree of decrease indicating caution. As already mentioned, the significance of any particular degree of depression depends on the rapidity with which it is reached.

A kit that is practical for measuring blood cholinesterase activity under field conditions has been developed by Edson.[19]

Protective Devices

Certain aspects of protective devices were mentioned above in connection with a discussion of the importance of conditions of use. It remains to say that many people who are familiar with the importance of these devices do not know that masks and respirators must be chosen separately for each compound. Our knowledge of satisfactory equipment depends on extensive and continuing research by Fulton and his co-workers.[25]

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TOXICOLOGY PROGRAM
Technology Branch
Communicable Disease Center
Atlanta 22, Ga.

CHLORINATED HYDROCARBON PESTICIDES IN THE FAT OF PEOPLE IN NEW ORLEANS

Wayland J. Hayes, Jr., William E. Dale and Virlyn W. Burse
U. S. Department of Health, Education, and Welfare
Public Health Service
Communicable Disease Center
Technology Branch, Toxicology Section
Atlanta, Ga. 30333

Wildlife section

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The Mississippi River and its tributaries serve as a drainage system for surface water for a large part of the United States. A recent report[1] attributed fish kills which occurred between October 1963 and February 1964 in the lower Mississippi River to minute quantities of endrin in the fish. This same report states that dieldrin and endrin are both present in the drinking water of the city of New Orleans and that dieldrin, endrin, heptachlor, DDT, and DDE have all been found in shrimp from the Gulf of Mexico.

The storage of chlorinated hydrocarbon pesticides in the fat of people without occupational exposure is well known.[2-15] In view of the above report[1] the following study was conducted to determine the concentration of these materials in the fat of people in New Orleans.

METHOD

Twenty-five autopsy samples of human fat were received in July 1964 from the Department of Pathology, Louisiana State University School of Medicine (Charity Hospital). The samples were taken from people ranging in age from 41 to 97 years. Of the 25 samples collected, 13 were from males. There was no known occupational exposure to pesticides, and in no instance was death or illness due to pesticides. The samples were preserved by refrigeration during shipment.

Details of extraction, clean-up, and preparation of samples for gas chromatography identical to those used in this study have been published elsewhere.[7] The gas chromatographic analyses were carried out with a MicroTek 2503-R instrument equipped with a Dohrmann Microcoulometric Detector. Each column consisted of an aluminum tube 10 feet by 3/16 inch O.D., and both polar (QF-1) and non-polar (Dow-11) column packings were used in separate columns. Details of the column packings and the retention times,

calculated relative to aldrin as unity, for various chlorinated pesticides have been published.[16] The method used determines quantitatively 0.1 μg of each of the chlorinated hydrocarbon pesticides reported in Table I.

RESULTS

Statistical evaluations of results reported in the table showed that the people of New Orleans had significantly more BHC ($p < 0.001$), dieldrin ($p < 0.01$) and p,p'-DDE ($p < 0.005$) in their fat than people of other parts of the United States[8], namely, Wenatchee, Washington; Phoenix, Arizona; and Louisville, Kentucky. The average concentration of heptachlor epoxide was 2.4 times as much as that reported[15] for 64 samples of fat from people from the four geographic regions of the country. The mean concentrations of o,p'-DDE and p,p'-DDD were 0.29 ± 0.03 ppm and 0.45 ± 0.05 ppm, respectively. These two materials have not been reported previously for the general population of the United States. The concentration of o,p'-DDT was 0.06 ± 0.03 ppm and is significantly less ($p < 0.005$) than that reported[8] for other sections of the country. There was no significant difference in the storage of p,p'-DDT in the fat of people in New Orleans as compared with other parts of the country.

An average of 77% of the total DDT-derived material was present as the less toxic ethylene derivative, DDE.

A comparison between 13 males and 12 females was made for the storage of all the pesticides reported in the fat of people in New Orleans. It was found that the females stored significantly more beta BHC ($p < 0.05$) and p,p'-DDT ($p < 0.02$) than the males. A comparison between 13 persons of the Negroid race with 12 Caucasians showed that there was no significant difference in the storage of any of the compounds reported in the fat of people in New Orleans based on race.

Endrin which was reported[1] in the fish and water of the Mississippi River was not found in the fat of the people of New Orleans nor has it been reported in the fat of the general population of this or any other country. However, endrin is capable of storage, having been found in fish[1], dosed rats[17] and the tissues of an accidentally exposed man.[18]

With the exception of o,p'-DDT and p,p'-DDT, there were significantly higher concentrations of all of the chlorinated pesticides in the fat of people in New Orleans as compared with concentrations in fat samples from other parts of the United States. However, there was significantly less BHC ($p < 0.001$), p,p'-DDE ($p < 0.005$), o,p'-DDT ($p < 0.01$), p,p'-DDD ($p < 0.001$) and p,p'-DDT ($p < 0.05$) than was found in the fat of the general population of Delhi, India.[19] The people of New Orleans did have significantly higher concentrations of dieldrin ($p < 0.001$) in their fat than were found in the fat of people from Delhi. Heptachlor epoxide and o,p'-DDE, which were reported in the fat of people of New Orleans, were not found in the fat of people of India.

Table 1

Concentration of Chlorinated Pesticides in the Body Fat of People in New Orleans, Louisiana Compared with that of People in Other Parts of the United States and Delhi, India all Measured by Microcoulometric Gas Chromatography and Expressed as Parts Per Million.

COMPOUND	STATISTICAL VALUE	LOCATION		
		New Orleans (25 samples)	Wenatchee, Wash.[8] Phoenix, Ariz. Louisville, Ky. (28 samples)	Delhi, India[19] (24 samples)
p,p'-DDT	Range	0.15-5.99	0.09-4.97	1.3-51.3
	Mean±S.E.	1.73±0.26	1.14±0.21	13.5±2.4
o,p'-DDT	Range	< 0.01-0.67	0.09-8.24	< 0.05-4.1
	Mean±S.E.	0.06±0.03	1.30±0.35	0.79±0.25
p,p'-DDE	Range	0.56-21.17	0.48-10.40	2.0-23.0
	Mean±S.E.	6.85±0.8	3.82±0.54	11.6±1.2
o,p'-DDE	Range	0.05-0.53		
	Mean±S.E.	0.29±0.03		
p,p'-DDD	Range	0.09-0.99		0.5-6.5
	Mean±S.E.	0.45±0.05		2.7±0.4
Total as DDT	Range	1.10-27.69	0.61-17.39	6.60-71.5
	Mean±S.E.	10.32±1.10	6.69±1.02	30.2±3.8
DDE as DDT	Percent	77	62	43
α-BHC	Range	< 0.03		< 0.03-0.77
	Mean±S.E.			0.28±0.37
β-BHC	Range	0.03-2.43		0.17-3.16
	Mean±S.E.	0.60±0.10		1.39±0.17
Total BHC	Range	0.03-2.43	< 0.03-0.70	0.20-4.9
	Mean±S.E.	0.60±0.10	0.20±0.04	1.7±0.23
Heptachlor epoxide	Range	0.03-1.45		
	Mean±S.E.	0.24±0.05		
Dieldrin	Range	0.03-1.15	0.02-0.36	< 0.02-0.36
	Mean±S.E.	0.29±0.05	0.15±0.02	0.03±0.01
Endrin	Range	< 0.03		

S.E. = Standard Error

Although the general population is exposed to BHC, which consists of a mixture of isomers (alpha, 65 to 70%; beta, 6 to 8%; gamma, 12 to 15%; delta, 2 to 5%; epsilon, 3 to 7%; and other isomers, 2 to 3%[30]), only the beta isomer was present in the fat of people of New Orleans. Both the alpha and beta isomers were present in the fat of the people of India, the beta isomer being present at 4.9 times the concentration of the alpha isomer. This was not unexpected because Davidow and Frawley[21] found that rats store a higher percentage of the beta isomer as compared with the alpha and other isomers.

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P. O. Box 769, Savannah, Ga.

Storage of DDT and DDE in People with Different Degrees of Exposure to DDT

WAYLAND J. HAYES Jr., M.D., Ph.D., Savannah, Ga.; GRIFFITH E. QUINBY, M.D., M.P.H., Dugway, Utah; KENNETH C. WALKER, B.S., Yakima, Wash.; JOSEPH W. ELLIOTT, M.S., Savannah, Ga., and WILLIAM M. UPHOLT, Ph.D., San Francisco

Extensive use of the insecticide, DDT [2,2 bis-(*p*-chlorophenyl)-1,1,1, trichloroethane], has resulted in varying exposure of those engaged in its manufacture, formulation, packaging, or application. Although people with occupational exposure are numerous, they form only a small fraction of the population which is potentially exposed to residues in food. Evidence for the widespread occurrence of dietary exposure was provided by Walker and his associates,[12] who found small but measurable amounts of DDT and its metabolite, DDE [2,2 bis-(*p*-chlorophenyl)-1,1 dichloroethylene], in all meals which they analyzed.

Howell[6] reported the storage of DDT-derived material at a concentration of about 17 parts per million (ppm) in the fat of a man with occupational exposure for four years and a history of eating foods known to bear appreciable residues.

Perry and Bodenlos[10] were unable to detect DDT in fats from 16 applicators whom they studied. Only a little later, however, Laug et al.[8] demonstrated DDT ranging from 0.0 to 34 ppm (mean 5.3 ppm) in the fat of 75 persons with no special occupational exposure. They found no sex difference in regard to the storage of DDT in adipose tissue but did find as high as 0.77 ppm (mean 0.13 ppm) in human milk. Mattson et al.[9] presented evidence that DDE was deposited in human fat in slightly larger quantities than was DDT. Hayes et al.[5] found from 3 to 13 ppm of DDT (mean 7.4 ppm) and from 5 to 26 ppm of DDE (mean 11.2 ppm) in the fat of human volunteers before the men began experimental exposure.

A general review of the relevant literature[4] makes it clear that fat is the most important tissue for the storage of DDT. This is not surprising in view of the known solubility of the compound in fatty solvents and its practical insolubility in aqueous solvents. The storage level of DDT in the fat does not increase indefinitely during continuous exposure but rather reaches a plateau characteristic of any particular level of intake. Not all species show the same concentration of storage when maintained on the same dosage. At any substantial level of intake, female rats store more DDT-derived material than do males, but no difference is apparent at low levels of intake. A sex difference in the storage of DDT has not been demonstrated in other species.

Study of groups of men who voluntarily ingested DDT at the rate of 3.5 to 35 mg/man/day, respectively, showed that they reached equilibrium of DDT storage within a year.[6] The level of storage corresponded to dosage. No sex difference has been noted in human storage of DDT. Whereas a sex difference in this regard appears unlikely, it must be recalled that there has been no opportunity to study higher dosage levels in women.

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From the Technical Development Laboratories, Communicable Disease Center, Bureau of State Services, Public Health Service, U. S. Department of Health, Education, and Welfare, Savannah, Ga., and Wenatchee, Wash. Medical Director (Dr. Hayes), Senior Surgeon (Dr. Quinby), Chemists (Mr. Walker and Mr. Elliott), and Senior Scientist (Dr. Upholt).

Although much is known about the storage of DDT and DDE in man, there has been no systematic survey to determine the storage of these materials in groups of people with different degrees of dietary and of occupational exposure to DDT. It seemed desirable that such a survey be made.

Materials and Methods

In order to obtain a base line, autopsy samples taken and sealed before the advent of DDT were obtained from the University of Louisville Medical School.

Biopsy samples of human fat were collected in 1954, 1955, and to a small extent in 1956. The biopsies taken were incidental to corrective surgery.

Fat tissue from persons whose relative abstinence from meat indicated a minimal dietary exposure to DDT were obtained from the Walla Walla General Hospital, Walla Walla, Wash.

Biopsies from persons with ordinary dietary habits and no occupational exposure to insecticides were obtained chiefly from the Public Health Service Hospital, Savannah, Ga., and from The Deaconess Hospital, Wenatchee, Wash. The latter hospital also furnished many samples from persons with incidental or occupational exposure to DDT.

In addition to the biopsies, samples were obtained from autopsies performed during the same period, 1954-1956. The autopsy material permitted a comparison of DDT storage in various tissues and in adipose tissue taken from different parts of the body. The recent autopsy samples were obtained through the kindness of the Seattle-King County Coroner, Seattle, and the Chelan County Coroner, Wenatchee.

In all instances, the size of fat samples sought was 2 gm. or larger.

For the planned survey, each fat sample was blotted with gauze or paper toweling, weighed to the nearest 0.01 gm., and stored in CP-grade carbon tetrachloride to await analysis. In most instances in which it was possible to obtain a sample weighing 4 gm. or more, the sample was subdivided. One portion was weighed and stored in carbon tetrachloride in the usual way, while other portions were weighed and stored in 10% formaldehyde, or frozen. These procedures permitted a comparison of the effects of different methods of sample storage on sample weight and ultimately on reported content of DDT-derived material.

Those adipose and other tissues taken in autopsies at considerable distances from the chemical laboratory were frozen for convenience in storage and transportation. These samples were only partially thawed before subdivision and weighing.

After each biopsy specimen was procured, a history of exposure to DDT was obtained. The patient's place of residence was recorded, particularly with regard to its location near factories or farms where insecticides were made or applied. If the patient had contacts with DDT in the home, garden, or elsewhere, the frequency and duration of exposure and the character of the formulation were recorded. In certain hospitals treating patients with special dietary practices peculiar to their religious group, a careful dietary history was obtained. Each case was then classified according to its history and placed in one of the following categories, arranged in order of increasing exposure:

1. Persons who died before the advent of DDT in the United States (1942) (10 persons).

2. Abstainers from meat without significant environmental or any occupational exposure (16 persons). This group contained no true vegetarians. Several of the subjects abstained from eating poultry or fish but did eat dairy products and eggs. Most of them ate both poultry and fish but partially abstained from mammalian flesh. Subjects were included in this classification if they admitted eating mammalian meat less than twice a month (Group 2a) or from 2 to 12 times per month (Group 2b) for at least two years.

3. General population with ordinary dietary habits and little or no environmental exposure (61 persons). These were persons living in various parts of the country who admitted no more than ordinary use of DDT in the household, garden, or place of work and who lived more than a mile from an orchard or other source of environmental exposure and who did not frequent such places with any regularity.

4. People with ordinary dietary habits and significant environmental exposure (110 persons). This group consisted of people living or working within one mile—and frequently very much less—of orchards or farms of North Central Washington so that their environment was exposed to many heavy applications of DDT per year.

5. Agricultural applicators with occupational exposure to DDT but not within one year of sampling (11 persons).

6. Agricultural applicators with occupational exposure to DDT within one year of sampling (19 persons).

Forty-five of the survey samples were subdivided and a portion used for other tests. In addition, 53 samples of fat and 17 samples of various organs were obtained from 17 other persons for use in studies not concerned directly with the survey.

Eleven meals (two breakfasts, six lunches, and three suppers) were obtained from the cafeteria of Walla Walla College, College Place, Wash.

TABLE 1.—*Storage of DDT and DDE in the Fat of People in the United States as Determined by Degree of Dietary, Environmental and Occupational Exposure*

Exposure Group		No. Cases	Value and Unit		DDT		DDE	
No.	Description				Tissue	Extract	Tissue	Extract
1	Died before DDT	10	Range,	ppm	Not greater than reagent blanks			
2	Abstainers from meat	16	Range,	ppm	0-7	0-10	0-9	0-12
			Mean ± SE,	ppm	2.3 ± 0.44	3.5 ± 0.63	3.2 ± 0.63	4.9 ± 0.84
			Coef. var.	%	19	18	20	18
2a	Eat meat <2 ×/mo.	12	Range,	ppm	0-7	0-10	0-8	0-12
			Mean ± SE	ppm	2.1 ± 0.54	3.3 ± 0.81	2.8 ± 0.65	4.3 ± 0.98
			Coef. var.	%	26	25	23	23
2b	Eat meat 2-12 ×/mo.	9	Range,	ppm	2-5	3-6	2-9	4-12
			Mean ± SE	ppm	2.8 ± 0.76	4.0 ± 0.71	4.5 ± 1.6	6.8 ± 1.9
			Coef. var.	%	27	18	36	28
3	General population	61	Range,	ppm	2-12	3-22	2-13	3-25
			Mean ± SE	ppm	4.9 ± 0.35	6.8 ± 0.42	6.1 ± 0.42	8.6 ± 0.52
			Coef. var.	%	7	6	7	6
4	People with heavy env. exposure	110	Range,	ppm	2-14	2-18	2-25	0-34
			Mean ± SE	ppm	6.0 ± 0.26	7.9 ± 0.41	8.6 ± 0.48	11.0 ± 0.62
			Coef. var.	%	4	5	6	6
5 + 6	Agricultural applicators total	30	Range,	ppm	3-37	6-48	4-58	4-95
			Mean ± SE	ppm	14.0 ± 1.49	20.5 ± 2.4	19.0 ± 2.2	27.3 ± 4.2
			Coef. var.	%	11	12	12	15
5	More than 1 yr. ago	11	Range,	ppm	4-16	6-46	4-21	10-39
			Mean ± SE	ppm	8.8 ± 1.03	16.1 ± 3.2	13.4 ± 1.46	22.4 ± 2.6
			Coef. var.	%	12	20	11	13
6	Within last yr.	19	Range,	ppm	3-37	9-48	4-58	4-95
			Mean ± SE	ppm	17.1 ± 2.1	23.1 ± 3.3	22.3 ± 3.0	30.2 ± 6.5
			Coef. var.	%	12	14	13	22

These meals were considered typical of those eaten by meat abstainers.

All analyses reported in this paper were carried out by the Schechter-Haller spectrophotometric method. The modifications described by Chemical Memorandum No. 1 [11] were used for tissue samples. Food samples were processed using the modifications of Walker et al.[12]

For statistical treatment, all samples which produced recognizable Schechter-Haller colors were accepted at their calculated values even though the limited accuracy of the method for "trace" quantities of 1μg. to 5μg. was recognized. Samples which gave no visible blue, red, or intermediate color were treated as zero, being beneath the experimental limits of the method.

Results

Storage of DDT-Derived Material in Man as Related to Exposure.—It is clear from Table 1 that the storage of DDT and of DDE in people varies according to their dietary and occupational exposure.

Analysis of 10 samples of human fat collected and sealed before the advent of DDT in this country failed to develop a color typical of DDT or any known metabolite. Although some of the samples produced yellowish or brownish colors, the spectrophotometric readings did not exceed the readings taken on reagent blanks processed at the same time but without any fat tissue. Thus, data which will be presented elsewhere indicate that, under the conditions now in use, the lipids extracted from human adipose tissue actually reduce the amount of light-absorbing material passing through a Davidow column. In any event, the results confirm the conclusion reached by Mattson et al.[9] that human fat does not spontaneously contain any material which might be confused with DDT in the Schechter-Haller reaction.

Abstainers from meat stored, on the average, about half as much DDT and half as much DDE as persons in the general population who stored an average of 4.9 ppm

TABLE 2.—*Statistical Significance of Differences in DDT Storage in Human Fat Associated with Degree of Exposure*

Comparison		Level of Significance, %	
Description of Exposure	Groups	DDT	DDE
Meat abstinence vs. ordinary diet	2 vs. 3	>99	>99
Meat abstinence <2 X/mo. vs. 2-12 X/mo.	2a vs. 2b	<50	<80
Little environmental exposure vs. heavy environmental exposure	3 vs. 4	>98	>99
Heavy environmental exposure vs. agricultural use within yr.	4 vs. 6	>99.9	>99.9
Agricultural exposure more than 1 yr. ago vs. agricultural use within yr.	5 vs. 6	>99	>95

and 6.1 ppm of the two compounds respectively. Consideration of fat samples from 16 meat abstainers and samples from 61 persons in the general population with little or no environmental exposure showed the difference to be statistically significant (Table 2) as regards both DDT and DDE. Twelve persons who admitted eating meat less than twice a month stored slightly less DDT and DDE than did four persons who ate meat from 2 to 12 times per month, but the difference was not statistically significant ($P>0.2$). The results of studies on meatless meals are given later.

Persons in the general population with significant environmental exposure (Group 4) stored, on the average 6.0 ppm of DDT, while those with little or no environmental exposure (Group 3) stored an average of 4.9 ppm. Although the difference was small it was statistically significant ($P<0.02$).

Agricultural occupational exposure caused a much more marked increase in the storage of DDT-derived material. The increase above general population levels was especially marked in persons who had had occupational exposure within a year (Group 6). These people stored DDT at an average concentration of 17.1 ppm.

Storage of DDT-Derived Material in Man as related to Age, Sex, Duration of Exposure, and Other Factors.—No evidence of differences in the storage of DDT-derived material due to age or sex were uncovered in this study.

Of the samples from the general population with little or no environmental exposure (Group 3), 50 were obtained in Savannah and 11 were obtained in Wenatchee. The histories indicated that many of the people did not live in the city in which they were hospitalized. Furthermore, some of them had moved from one part of the country to another in recent years. In spite of these facts, it is of interest that the difference in DDT storage between the two groups was small and of questionable statistical significance ($P>0.05$).

Considering all the samples in the survey, DDE averaged 58% of the DDT-derived material. This matter is covered more completely in the comment.

The Distribution of DDT and DDE within various tissues of the body.—Table 3 indicates that similar concentrations of DDT were found in samples of fat taken from the same person regardless of the location of the sample.

In the limited autopsy material available, the concentration of DDT was below the experimental limits for the method for brain, liver, and ovary tissues. Carbon-tetrachloride-extractable material constituted 11% to 30% of 11 samples of adrenal glands. In every instance, a trace of DDT (average 3.4 ppm based on extractable lipid) was detected in the adrenals. (If all DDT in the adrenals were in neutral fat and this fat constituted 54% of the total lipids, as reported by Bloor[2] for guinea pigs, then the concentration of DDE in the neutral fat of the adrenals would be 6.3 ppm.) Peri-adrenal fat taken from 9 of the 11 subjects showed 64% to 80% extractable lipid and an average concentration of DDT of 7.5 ppm in the lipid.

Concentration of DDT-Derived Material in Fresh Adipose Tissue and in Extractable

TABLE 3.—*Concentration of DDT in Fat from Different Anatomic Sites*

Person	Sample (see text)	Anatomic Site: Subcutaneous (Ppm)	Mesenteric (Ppm)	Retroperitoneal (Ppm)	Pericardial (Ppm)	Intramuscular (Ppm)	Retrobulbar (Ppm)	Mean ± SE (Ppm)	V x̄ (%)
1	Adipose	2.9	2.8					2.9	
2	Adipose	2.0 — 2.4[4]	2.1 — 2.7[3]	1.9 — 2.2[3]	1.8		1.2	2.0 ± 0.12	6
3	Adipose	2.3 — 2.8[3]	2.6 — 2.7[3]	2.4	2.1			2.5 ± 0.09	4
4	Adipose	6.1 — 6.3[2]		4.5				5.6 ± 0.57	10
5	Adipose	6.7 — 8.8[3]			2.8			6.4 ± 1.3	20
6	Adipose	8.5 — 11.2[3]	9.8[3]	9.4[3]	18.2	10.4		10.8 ± 0.96	9
7	Adipose		14.9	8.2 — 12.9[3]	10.8			11.7 ± 1.4	12
8	Adipose	22.2 — 26.3[3]	16.2 — 17.9[3]	18.8 — 27.4[3]	23.3			22.5 ± 1.2	5
1	Lipid	3.4	3.4					3.4	
2	Lipid	2.6 — 3.4[4]	3.2 — 3.3[3]	2.9 — 3.2[3]	2.4		2.0	2.8 ± 0.13	5
3	Lipid	2.9 — 3.2[3]	3.0 — 3.3[3]	3.0	2.8			3.0 ± 0.35	8
4	Lipid	6.3 — 7.0[2]		6.3				6.5 ± 0.25	4
5	Lipid	11.3 — 14.1[3]			16.7			13.8 ± 1.1	8
6	Lipid	14.8 — 15.5[3]	13.8 — 14.7[3]	13.8 — 15.2[3]	28.8	15.1		16.3 ± 1.6	10
7	Lipid		27.8	17.4 — 18.2[3]	17.6			20.3 ± 2.5	12
8	Lipid	29.8 — 36.0[3]	28.8 — 30.5[3]	26.5 — 34.6[3]	32.9			32.3 ± 1.0	3

Superscripts represent number of samples where greater than one. No two Samples were taken from the same location. For example, subcutaneous samples came from the axilla and breast as well as various positions on the abdomen.

Lipids.—The concentration of DDT in the tissues of man and animals has generally been expressed in terms of fresh tissue weight. The concentration in cows' milk has frequently been expressed in terms of butterfat or in terms of milk of standard butterfat content.

The amount of carbon-tetrachloride-extractable lipid was determined for 216 samples of fat by weighing the residue remaining after evaporation of an aliquot (usually 1/10) of the carbon tetrachloride extract of the entire sample. The concentrations of DDT and DDE were reported in terms of both fresh weight and extractable lipid. The extractable material varied from 9% to 95% of the fresh weight of the corresponding samples. As shown in Table 4, 5% of the samples contained less than 50% of lipids. About two-thirds of the samples contained between 70% and 89% of lipids.

The few samples containing less than 50% of lipids frequently gave difficulty. In the first place, samples of this sort from the general population have to be large in order to have enough DDT-derived material present to permit its detection. Secondly, these samples frequently gave, on complete analysis, colors ranging from chrome yellow to dark amber or brown. It may be that the materials responsible for these colors are more abundant in connective tissue than in fat per se.

Occurrence of DDT and DDE in Whole Meals.—Eleven meatless meals were analyzed portion by portion. Of the 66 individual items analyzed, only 9 contained detectable DDT and only 3 contained over 6 μg. of DDT. Each of the latter three dishes contained eggs in one form or another. The mean daily DDT content of the meatless diet was determined to be 0.041

TABLE 4.—*Proportion of Samples of Adipose Tissue Showing Different Concentrations of Extractable Lipids*

Proportion of Fresh Tissue Weight Extractable by CCl_4 (%)	Proportion of Samples (%)
0-49	5
50-59	6
60-69	19
70-79	31
80-89	33
90-99	6
0-99	100

mg., which may be compared with 0.184 mg., the average determined in a similar way in the same laboratory for ordinary restaurant meals.[12] Vegetables showed low DDT residues both in the meatless meals and in the restaurant meals. The meatless meals contained 36% of the DDT-derived material in the form of DDE, a value essentially identical with that for ordinary meals.

Effect of Various Modes of Preservation on the Apparent DDT Content of Fat Samples.—It is a common practice to preserve fat samples for organic chloride insecticide analysis in CP-grade carbon tetrachloride, and this method was taken as standard for the present study. In this procedure, the sample is weighed while fresh. However, specimens preserved by formaldehyde or freezing are frequently received for analysis. Without some study, such samples pose a problem if the results are to be given in terms of concentration of DDT in fresh tissues.

The change of weight of fat tissue caused by storing it for 20 or more days in 10% formaldehyde or by freezing is shown in Table 5. For periods as great as 103 days, storage in formaldehyde did not change the fresh weight of fat more than 8%. The changes were in both directions and averaged essentially zero (+0.2%). This small variation in weight can be attributed mostly to unavoidable variation in the degree of blotting of tissues before weighing. Even the maximum variation encountered (8%) does not exceed the variation in chemical results frequently encountered between two fat samples taken at the same time from the same body (Table 3).

TABLE 5.—*Weight Change of Adipose Tissue Associated with Storage in 10% Formaldehyde or by Freezing*

Value	Formaldehyde (23 Samples)		Freezing (7 Samples)	
	Days	% Change	Days	% Change
Range	21–103	−8 to +5	20–63	−13 to −3
Mean		+0.2		−8.3

Twenty-two paired subsamples of fat preserved in carbon tetrachloride and formaldehyde respectively were analyzed for DDT-derived material. Fourteen of the pairs differed less than 1 ppm in DDT content. Six of the pairs deviated 1 ppm in either direction. The two remaining pairs deviated 2 ppm and 4 ppm respectively in opposite directions. Variations of a similar magnitude in both directions also occurred for DDE. The lack of a significant difference was further demonstrated by the use of "Student" *t*-test ($P>0.5$ for both DDT and DDE). In a similar way, the same samples showed no significant difference in the percentage of extractable lipid.

On the other hand, freezing consistently led to loss of weight of the samples, owing to drying. The observed losses ranged from 3% to 13% and averaged 8.3%.

Comment

There apparently has been no progression in the storage of DDT in the general population of the United States since this storage was first measured in 1950. Evidence leading directly to this conclusion is given by comparing the results of studies made in different years. As shown in Table 6, the median storage has remained essentially the same. It may even be possible to explain the small variation in this median between one study and another. Thus the men reported by Hayes et al.[5] tended to store a little more DDT than those reported here. This may have been associated with the fact

TABLE 6.—*Range of Concentration of DDT Found in the Fat of People with Ordinary Diet and No Occupational Exposure*

Range (Ppm DDT)	Cases, Per Cent			
	Laug et al. (1955)	Hayes et al. (1956)	This Study	
			Group 3	Group 4
0.0	20	0	3	1
0.1–1.0	9	0	0	1
1.1–5.0	28	25	69	45
5.1–10.0	28	61	25	45
10.1–20.0	12	14	3	8
Over 20.0	3	0	0	0

that their daily DDT intake, as measured by the analysis of whole meals, was slightly higher than that for the general population[5] (0.202) as compared with 0.184 mg/man/day.[12]

In a study of ordinary restaurant meals, Walker and his associates[12] discovered that "generally those foodstuffs cooked in fat, and containing meat, meat combinations, or butter, were found to have a higher DDT content than [other] foodstuffs . . ." This report is entirely consistent with the finding of only very small quantities of DDT in "meatless" meals and the storage of only very small concentrations of DDT-derived materials in the adipose tissue of meat abstainers, as reported in this paper. In fact, the greater part of the DDT found in "meatless" meals was associated with poultry and other animal products.

In 50 samples of human fat, including some from persons having known high exposure to DDT, Mattson and his colleagues[9] found that in individual samples, DDE constituted from 39% to 86% and averaged about 60% of the total DDT-derived material. In the present study, the corresponding range was 33% to 90% (excluding two samples in which no DDE was detected) with an average of 58%. The average for the different groups studied varied from 56% to 60%. Men reach an equilibrium of DDT storage within a year. The period necessary for reaching equilibrium of DDE storage may be similar although it is not so well established.[5] The fact that about 54% of the DDT-derived material in the general diet is DDE[12] may have some bearing on the proportion in which DDE is stored in the fat. On the contrary, DDE constitutes only a very small fraction of the DDT-derived material to which certain workers are exposed. It is therefore not astonishing that people with heavy exposure tend to store a lower proportion of DDE than do people in the general population. And yet, as shown in Table 7, persons with the greatest exposure may store 40% or more of their DDT-derived material in the form of DDE. Further study of the table suggests that duration of exposure is the greatest single factor in determining the relative amount of DDE stored in those exposed to comparatively great dosages of DDT and small dosages of DDE.

Table 7 indicates that agricultural applicators store far less DDT than certain formulators. However, agricultural applicators stored, on the average, about three times as much DDT and DDE as their neighbors with comparable environmental exposure. This implies that the exposure of these groups differed by a factor probably greater than three but less than six. We do not know enough to relate satisfactorily the exposure of applicators (as measured by Batchelor and his colleagues[1]) with the tissue storage which these men show. Certainly

TABLE 7.—*Percentage of DDE Associated with Different Concentrations of DDT*

Group Description	No.	No. Samples	Exposure (Yr.)	Storage of DDT (Ppm)	DDE * (%)	Authority
Abstainers from meat	2	16	9 †	0-7 (2.7 ± 0.40)	39-67 (58)	This paper
General population	3	61	9 †	2-12 (4.9 ± 0.35)	38-80 (56)	This paper
General population	--	49	7 †	3-13 (7.4 ± 0.37)	29-79 (60)	Hayes et al., 1956
General population and others	--	50	5 †	2-79	39-86 (60)	Mattson et al., 1953
People with heavy environmental exposure	4	110	9 †	2-14 (6.0 ± 0.26)	0-78 (57)	This paper
Agricultural applicators more than 1 yr. ago	5	11	8 †	4-16 (8.8 ± 1.03)	45-72 (60)	This paper
Agr. app. within yr.	6	19	9 †	3-37 (17.1 ± 2.1)	45-69 (56)	This paper
Volunteers	--	8	0.5	80-345 (202 ± 30.6)	8-29 (14)	Hayes et al., 1956
Formulator	--	1	6	122	49	Mattson et al., 1953
Formulator	--	1	5	684	40	Hayes et al., 1956

* DDE as percentage of total DDT-derived material.
† Estimated.

the applicators get a great deal of DDT on their skins, much more, in fact, than might seem necessary to account for the observed storage. The importance of inhalation of spray material may be discounted on the basis of measurements of parathion.[1] It is difficult to exclude the possibility that the applicators ingest some DDT in the process of eating, drinking, and smoking, though such ingestion has not been measured. Perhaps men absorb undissolved DDT through the skin as poorly as animals do.

Two conclusions seem justified: Much more study is needed to define the exact way in which agricultural chemicals enter the body under practical conditions of use. Secondly, there is an excellent chance that workers would absorb from the skin a much higher proportion of some other chlorinated hydrocarbons whose dermal absorption is less dependent on solution.

The hypothesis has been advanced[2,3] that DDT is stored in extractable lipids, whether in adipose or other tissues. At least in a general way, the known facts are consistent with this view.[4] Thus, it might be supposed that under any given conditions of exposure the variation of the reported concentration of DDT could be reduced by eliminating the variable associated with the proportion of extractable material in adipose tissue. However, the supposition was not confirmed by analysis of the data. The coefficient of variation was not appreciably smaller for concentrations expressed in terms of extractable lipid than for those expressed in terms of fresh weight. This was true whether one compared the results involving single samples from many persons (Table 1) or results involving several samples from different parts of the body of a single person (Table 3). The reason for the failure to confirm this biologically reasonable hypothesis is not clear.

For any systematic survey, the specimens should be taken and processed under as nearly identical conditions as possible. However, this study indicates that results for DDT storage obtained from fat samples preserved by formaldehyde are quite comparable to those obtained from samples preserved by carbon tetrachloride. The loss of weight caused by the drying of frozen samples leads to a systematic error if the samples are weighed after storage and if the results are expressed in terms of "fresh" tissue weight. However, even this error would not be of important magnitude—less than 14%.

The suitability of formaldehyde as a preservative for fats which are to be analyzed for compounds other than DDT has not been established.

Summary and Conclusions

During 1954-1956 samples of fat tissue from 227 people were analyzed for DDT and DDE and the results tabulated according to the dietary, environmental, and occupational exposure of these people to DDT. Also, 45 samples of fat from those surveyed as well as 70 samples of fat and other tissues from 17 other people were used for other purposes. Thus, 342 samples in all were analyzed.

There has apparently been no progression in the storage of DDT in the general population of the United States since this storage was first measured in 1950.

Persons abstaining from meat deposited in their fat only about half the concentration of DDT (2.3 ppm) as did people in the general population 4.9.

Meatless meals served in a cafeteria catering to meat abstainers contained only about a quarter as much DDT as did meals served in ordinary restaurants.

There is strong evidence that essentially all storage of DDT and DDE in persons without occupational exposure results from the presence of these compounds in the diet, especially but not exclusively in fats of animal origin.

The environmental exposures incidental to living or working in or near areas of heavy application of DDT caused deposits only very slightly greater than those in persons without such environmental exposure.

People with occupational exposure stored more DDT-derived material than other people and the rate of storage was generally proportional to the intensity and frequency of exposure.

DDE averaged 58% of the DDT-derived material stored in most people, but a smaller proportion in those with very great exposure. This finding is explained at least in part by the fact that about a third of the DDT-derived material in the ordinary diet is preformed DDE.

The results obtained from fat samples preserved in 10% formaldehyde are quite comparable with those obtained from samples preserved in carbon tetrachloride. Drying of frozen samples causes a systematic error if samples are weighed following storage, but the magnitude of the error is less than 14%.

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P. O. Box 769.

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Pathologic Changes in the Liver of Rats After Feeding Low Levels of Various Insecticides

ARNOLD J. LEHMAN, M.D., WAYLAND J. HAYES, JR., M.D., Ph.D., and WILLIAM F. DURHAM, Ph.D.
Savannah, Ga.

The chlorinated hydrocarbon insecticides, Chlordane,[1] Dieldrin,[2] Heptachlor[3] and Toxaphene[4] were chosen for study because they represent a group of economically useful poisons. Although human fatalities have resulted from massive exposure, most interest has centered on the possible cumulative effects of repeated low dosages which the general population may potentially obtain from residues in foods or, in the case of chlordane, from thermal vaporizers. The present report is a logical extension of a previously published paper on the pathology produced by D. D. T.[5] in the white rat.

Methods

Earlier work has defined the toxic limits in rats of the insecticides under study. Table 1 compares the dietary levels used in the present study with dietary levels reported in the literature to cause no effect or a minimal effect. The same Table indicates the number of rats fed different concentrations and the intervals at which they were killed in the present experiment.

The dosages used were selected in an attempt to approximate the minimal levels which would presumably cause tissue lesions. In addition to the chlordane (Velsicol 1068, Velsicol Chemical Corp., New York), the sample was analyzed to contain 85% chlordane and 15% related compounds.

1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo,exo-5,8-dimethanonaphthalene (Dieldrin) was the 95% gamma isomer. The sample used was supplied by The California Spray Chemical Corp., Richmond, Calif.

Heptachlor (unknown), a chlorinated camphene of approximate empirical formula $C_{10}H_5Cl_7$ containing 67% to 72% chlorine, was procured by the Hercules Powder Co., Wilmington, Del.

1,2-bis(p-Chlorophenyl)-2,2,1-trichloroethane

Table 1.—Comparison of Dietary Levels Used in the Present Study with Dietary Levels Reported in the Literature to Cause no Effect or Minimal Liver Change

Insecticide	Dietary Levels, ppm*	Present Experiment: No. Animals at Each Dietary Level: Male	Female	Present Experiment: Duration, Months	Present Experiment: Autopsies, Months	Summary of Literature: Highest Dietary Level That Caused No Change, ppm	Summary of Literature: Lowest Dietary Level That Caused Change, ppm
Chlordane	100, 25	6	6	24	2, 4, 6, 9, 12, 18, 24	25†	25†‡, 10†, 40†
Dieldrin	100, 25	6	6	24	2, 4, 6, 9, 12, 18, 24	25†	25†
Heptachlor	50, 100	6	6	24	2, 4, 6, 9, 12, 18, 24	0.25†, 5†‡, 10†‡, 50†	100†‡
Toxaphene	50, 100	6	6	24	2, 4, 6, 9, 12, 18, 24	—	100†
Controls	0.0	25	25	30, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24			

* Two-fifty is all the animals, including the controls, fed chlordane were D.D.T., as indicated by the number of rats compared in Group 100. Chemical analysis of samples of these showed that the concentration of D.D.T. in it never exceeded a trace of 0.5 ppm but was certainly below the sensitivity of the method.

† Animals under stress of cold at autopsy.

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D. D. T., they were graded subjectively and compared to the expected range of variation as established by controls using the methods reported earlier.[1]

Results

Morbidity and Mortality.—One female rat after [illegible] days of 25 ppm of Chlordane had a 17 [illegible] period of hypersensitivity which spontaneously remitted. Later, at death, this animal was negative for microscopic pathology. There were no spontaneous deaths in the Chlordane series.

One Dieldrin-treated female had a convulsion after 74 days' exposure to 25 ppm, regained her weight, and was found dead two [illegible] Death was attributed to Dieldrin poisoning. No microscopic studies were made. A male of the same dosage was observed to be nervous on the 112th day of exposure only; the nervousness remitted and [illegible] days later minimal liver lesions were found. Another male given 25 ppm showed observed symptoms of poisoning but no observed signs of intercurrent infection or toxicity.

Two of the animals treated with Endrine showed some degree of nervousness. One male rat with 50 ppm for 105 days in the diet convulsed for 48 hours, spontaneously remitted, and then was negative for microscopic change at death. One female was exposed to 100 ppm for 200 days, was convulsive for 3 days, spontaneously remitted, and 140 days later, at death, showed minimal liver lesions. There were no spontaneous deaths in the Endrine series.

No clinical symptoms attributable to Toxaphene were noted. One male rat with a dietary level of 50 ppm of this compound died of intercurrent infection as indicated by autopsy.

Liver Weight.—Liver weight, which was expressed as percentage of total body weight, showed no significant deviation from control values in any experimental group.

Food Intake and Body Weight.—Average daily food intake (calculated as grams of food per kilogram of body weight) and average cumulative body weight gain curves

TABLE 2.—*Summary of Histopathological Results Showing Ratio of Abnormal Animals to Total Examined*

Dose, p.p.m.	Sex	Chlordane, Type		Dieldrin, Type		Lindane, Type		Toxaphene, Type	
500		2/2	2/2	2/2	2/2	2/2	1/2	2/2	2/2
100	M	2/2	2/2	2/2	2/2	2/2	2/2	2/2	1/2
	F	2/2	2/2	2/2	2/2	2/2	2/2	2/2	1/2
50	M	2/2	1/2	2/2	2/2	2/2	2/2	2/2	2/2
	F	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
25	M	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
	F	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
10	M	2/2		2/2	2/2	2/2	1/2		
	F			2/2	2/2	2/2	1/2		
5	M	2/2	2/2					1/2	2/2
	F	2/2	2/2					1/2	2/2
Check	M	1/2	2/2	1/2	1/2	1/2	1/2	2/2	1/2
	F	1/2	2/2	1/2	1/2	1/2	1/2	1/2	2/2

for the experimental animals served as a uniform deviation from control value.

Pathology.—No gross disease was observed in either rats at necropsy. Table 2 indicates the incidence of moderate and severe hepatocellular injury, both sexes of exposure and the number showing significant injury of any degree. Only the liver of the animals showed consistent lesions which could be definitely related to chlorinated hydrocarbon exposure. Alterations such as pigment storage, hyaline droplet accumulation were found within renal tubules but proved indistinguishable from changes seen in controls. Likewise, minor fluctuations of the splenic lymphoid stroma were present.

The type of hepatic change observed with these compounds was identical to that seen at low levels of D. D. T. feeding. As exposure was increased in both dosage and duration, three stigmata of cellular disease were observed: (1) centrilobular-cell hypertrophy, (2) peripheral migration of basophilic cytoplasmic granulations, and (3) the presence of distinctive cytoplasmic inclusion bodies. The latter were called *lipospheres* for convenience in order to distinguish them from nonspecific hyaline or refractile cytoplasmic inclusions, which reflect the characteristic structure and staining reactions of lipospheres.

The control animals had considerable variation in hepatic storage of nonspecific lipoid and neutral fat droplets. Hence, these two kinds of droplets were not useful as indices of exposure to the hydrocarbons under test. The situation was quite different when the fatty substances existed as lipospheres, having a fibrillar capsule staining predominantly for phospholipid and a central more vacuolated zone staining characteristically for neutral fat. Previous studies had established that lipospheres were absent from control animals and present only when appreciable exposure to D. D. T. had occurred. A fairly distinctive staining reaction of these lipospheres with Sudan black B, Sudan IV, orange G, and aniline blue has after acid Zenker fixation made it practical to use these as an indication of liver-cell reaction to chlorinated hydrocarbon. It was feasible to use paraffin sections in screening for these fatty structures, for although the neutral fat moiety was extracted, the capsule stained brilliantly with acid fuchsin. Figures 1 to 9 illustrate typical examples of hypertrophy, margination, and lipospheres.

Several other liver-cell lesions were seen whose significance is difficult to evaluate. Two types of parenchymal bleeding were noted infrequently. The first, as demonstrated in Figure 9, consisted of macroscopic masses of red blood cells apparently actively dissecting the cytoplasmic substance of the liver cells. This type of involvement was confined to animals showing marked hypertrophy and margination, and it was seen in the lindane and Toxaphene series only. The second type, seen in Figure 10, had a more passive nonaggressive

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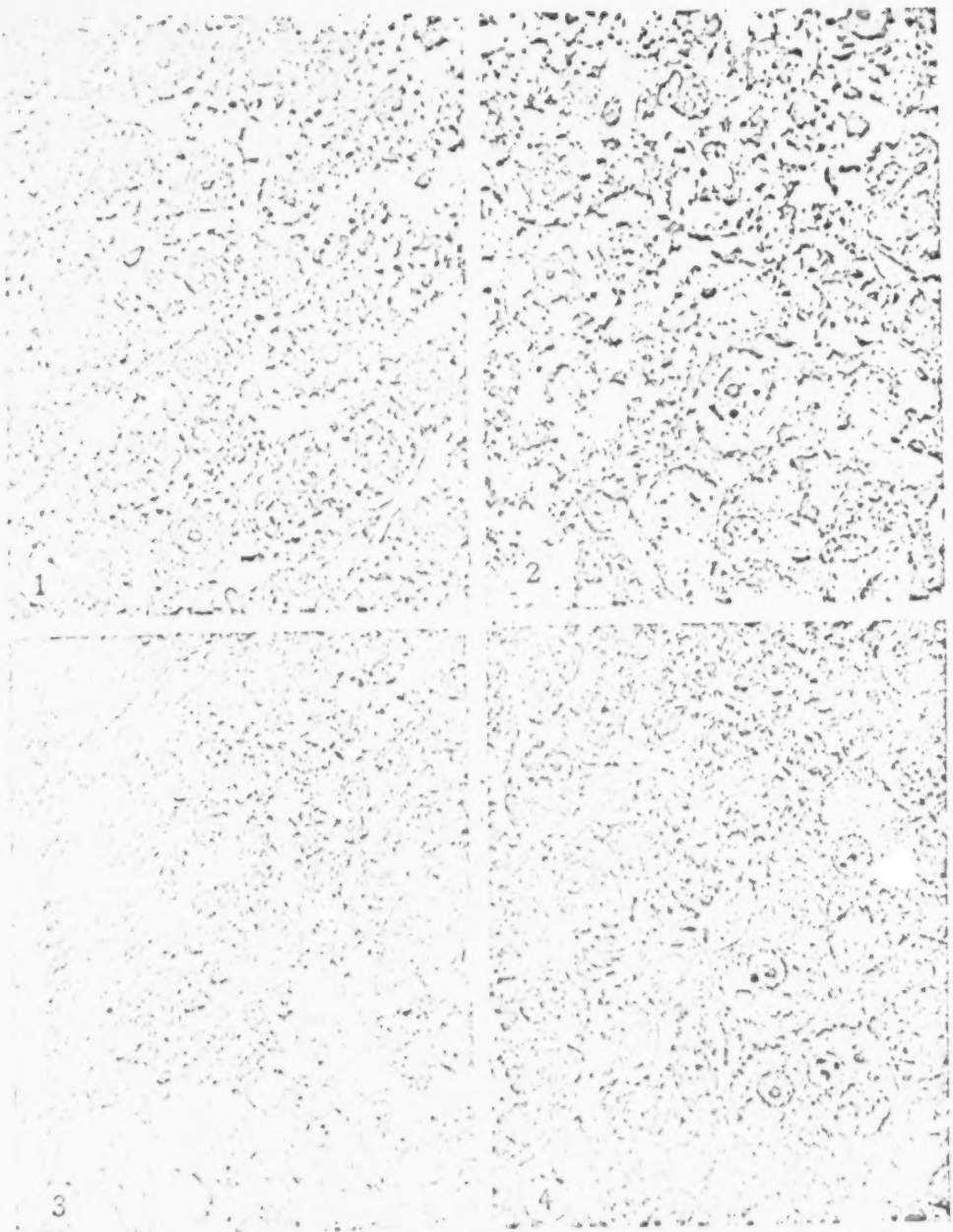

Fig. 1.—Normal rat liver cells, showing the usual pattern of cell sizes and cytoplasmic granules. Control diet, six months, male. Azure eosin after Zenker-formol fixation; × 1480.

Fig. 2.—Lower portion of picture shows liver cells with moderate degrees of margination and hypertrophy. Compare with Figure 1. Chlordane, 25 ppm, nine months, male. Azure eosin after Zenker-formol fixation; × 1480.

Fig. 3.—Inferiorly, a sharply demarcated mass of liver cells with marked margination and hypertrophy is contrasted with more normal cells superiorly. Toxaphene, 200 ppm, nine months, female. Azure eosin after Zenker-formol fixation; × 310.

Fig. 4.—Higher magnification of a group of cells similar to those in Figure 3. In addition to well-established hypertrophy and margination, cytoplasmic lipospheres can be dimly visualized as roughly circular outlines. Dieldrin, 25 ppm, four months, male. Azure eosin after Zenker-formol fixation; × 1220.

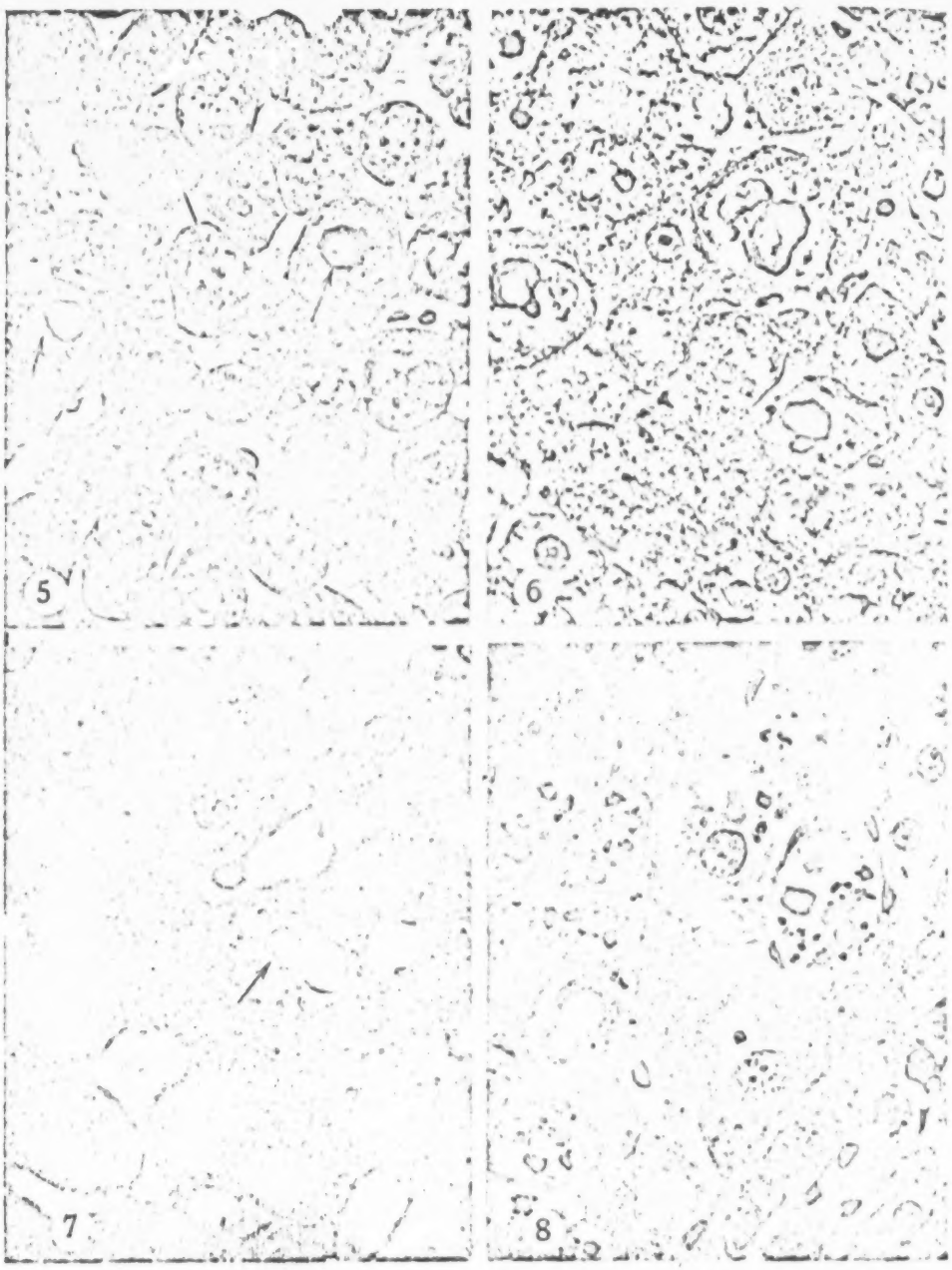

Fig. 5.—Group of liposphere. Small evolutionary forms . e seen in center above; larger densely stained body to left is associated with a hydropic vacuole. Large body to right of center shows a typically clear central zone having a delicate vacuolar network. Toxaphene, 200 ppm, four months, male. Fuchsin, aniline blue, orange G after acid Zenker fixation; × 1220.

Fig. 6.—In addition to cell enlargement and margination of cytoplasmic granules these liver cells also show many large bizarre lipospheres. The capsule of the lipospheres stains heavily with acid fuchsin, while the central area characterized by a complicated reticular

(Legend continued on next page)

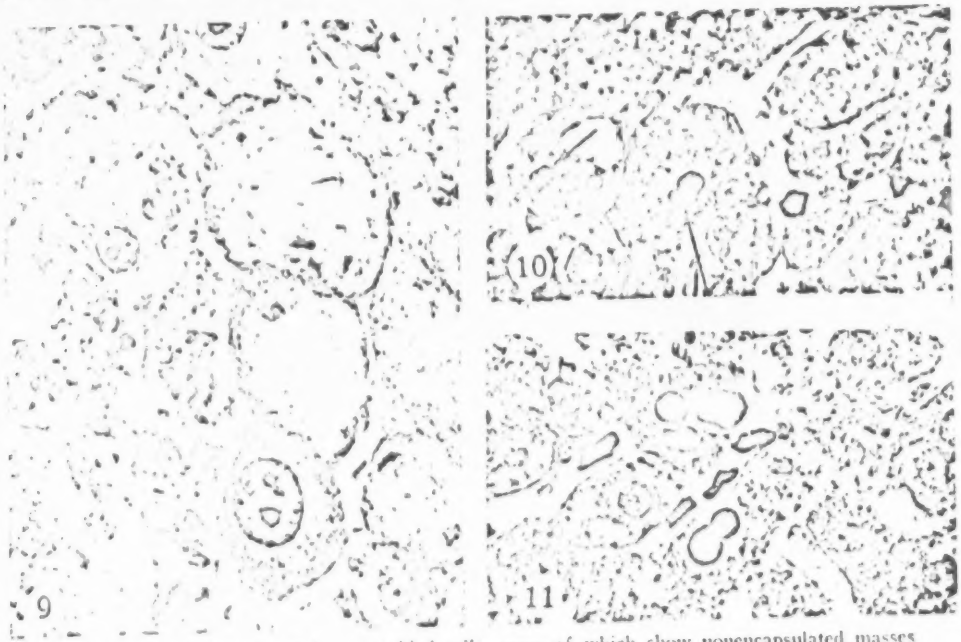

Fig. 9.—Marginated and hypertrophied cells, some of which show nonencapsulated masses of red blood cells with apparent cytoplasmic disruption. Lindane, 100 ppm, eight months, female. Azure eosin after Zenker-formol fixation; × 820.

Fig. 10.—The liver cell to the right shows a typical hydropic vacuole which contains two erythrocytes. The cell to the left contains a fatty vacuole and several small lipospheres. These hydropic changes have been observed after exposure to all chlorinated hydrocarbons tested. D. D. T., 5 ppm, two months, male. Fuchsin, aniline blue, orange G after acid Zenker fixation; × 1480.

Fig. 11.—A group of nonspecific waxy or hyaline bodies. Of note is the smooth regular contour of the amorphous uniformly staining substance. Chlordane, 25 ppm, nine months, female. Fuchsin, aniline blue, orange G after acid Zenker fixation; × 1480.

ruptive appearance and consisted of single, or small, groups of red blood cells lying within a cytoplasmic hydropic vacuole which had a distinct capsule or border. These vacuoles frequently were found within hepatic cells which were otherwise entirely normal, and only a minority of the vacuoles contained red blood cells. The vacuole was fat-negative and contained a finely granular material which invariably took the same stain as the surrounding intrasinusoidal plasma. The picture was one of plasma imbibition by liver cells, with the infrequent addition of red blood cells lying suspended within this plasma. This hydropic accumulation was observed in the Chlordane, Dieldrin, lindane, and Toxaphene series. It tended to be most prominent in male animals at the higher dosage levels, but the material was insufficient to confirm this suspected

structure remains largely unstained. Chlordane, 25 ppm, nine months, male. Fuchsin, aniline blue, orange G after acid Zenker fixation; × 1220.

Fig. 7.—Typical reaction of a single liposphere in center of field following traditional fat staining procedures. The densely positive center and fainter staining capsule are characteristic. Note that remaining portions of this and other cells are almost free of nonspecific fat positive globules. Chlordane, 25 ppm, two months, male. Oil red O after formalin fixation and carbowax imbedding, hematoxylin counterstain; × 1480.

Fig. 8.—Most of field is replaced by hypertrophied marginated cells containing both large and small lipospheres which are strongly fat-positive. Almost all of the fat-positive material is organized as lipospheres. A few cells of more nearly normal size are in the lower right corner. Toxaphene, 200 ppm, nine months, female. Oil Red O after fixation in formalin, mordanting in potassium dichromate, and paraffin imbedding, hematoxylin counterstain; × 1220.

relationship. These vacuoles on occasion were observed in close relationship to lipospheres, but it was not possible to categorize this as a casual or a chance occurrence. A similar experience was encountered with D. D. T.[1] where hydropic changes occurred erratically in cells of all types, while active bleeding was uncommon and confined to abnormal cells.

The hyaline or refractile spherical bodies mentioned above are pictured in Figure 11. They could not be related to poisoning, frequently being seen in control material. Although of theoretical interest, their only practical importance was that an inexperienced observer may mistake them for lipospheres. However, their complete lack of internal structure and their smooth contour, as well as their different reactions to a variety of stains, sharply distinguish them from lipospheres.

Comment

In general, results reported here are similar to those obtained by Lehman[2,3] and Patterson.[4] We are in agreement that careful microscopic examination of the liver cell will reveal cytoplasmic alterations at levels where biochemical and clinical examinations have so far failed to reveal any change, and the over-all consistency of these studies would tend to refute the suggestion by Cameron[9] that laboratory artifact is involved.

With sufficiently careful examination it is possible to detect, on occasion, liver cell abnormalities at lower levels of Dieldrin, lindane, and Toxaphene than those previously reported. Thus the lowest level which produces minimal tissue change can be reduced as follows: (1) Dieldrin, from 25 ppm to 2.5 ppm; (2) lindane, from 100 ppm to 50 ppm, and (3) Toxaphene, from 100 ppm to 50 ppm. In this regard some comment on the sensitivity of the method is in order. Of the total of 27 abnormal animals, 16 showed margination and/or hypertrophy of a degree not seen in control material. However, 11 of the 27 abnormal animals showed only lipospheres and would otherwise have been classified as normal. Only in the case of Toxaphene at 50 ppm in the male did the presence of lipospheres fail to equal or to exceed the sensitivity of margination or hypertrophy. It should be noted that at low levels of poisoning these inclusions are small and widely scattered and require optimal fixation, sectioning, and staining for study. The most precise results were obtained from considering all three types of lesions as indicated in Table 3, which summarizes the results.

Hydropic vacuolization of liver cells with the occasional presence of red blood cells is an interesting incidental finding; it has been reported for both toxic and physiological states.[10-13] Its pathological significance is not fully understood and any possible relationship of lipospheres, imbibed plasma or red cells, and hyaline bodies remains conjectural.

The general mildness of the liver-cell changes following exposure to repeated small doses of Chlordane, Dieldrin, lindane, and Toxaphene was apparent, and these changes were entirely similar to those following the ingestion of D. D. T.[1] The changes produced by D. D. T. were reversible, but reversibility was not tested for the other compounds. Although liver-cell changes have been found at new low levels of oral exposure to Dieldrin, lindane, and Toxaphene, Table 2 strongly suggests that each of these minimal

TABLE 3.—*Lowest Levels Producing Liver-Cell Abnormality*

Criteria for Diagnosis	Chlordane, Ppm		Dieldrin, Ppm		Lindane, Ppm		Toxaphene, Ppm	
	Male	Female	Male	Female	Male	Female	Male	Female
Margination and/or hypertrophy	25	>25	25	25	100	>100	50	200
Lipospheres alone	2.5	>25	2.5	2.5	50	100	200	50
Any combination of above 3	2.5	>25	2.5	2.5	50	100	50	50

vels exists at a threshold value, where only infrequent animal will be susceptible. The neral high resistance of the female is ilent. It would appear likely that as thods of cytological study are refined and creasingly subtle abnormalities disclosed, e physiological impairment becomes correspondingly reduced.

Summary

In order to reinvestigate the minimal ietary levels of certain chlorinated hydrocarbon insecticides required to produce athologic change, materials were fed to ats at the following concentrations: Chlorane, 2.5 and 25 ppm; Dieldrin, 2.5 and 25 pm; lindane, 50 and 100 ppm; and Toxahene, 50 and 200 ppm. The control diet ontained less than 0.6 ppm of D. D. T. as e only known contaminating insecticide.

The experimental animals were observed linically and then serially killed after from wo to nine months' exposure to the poison. Tissues from liver, kidney, and spleen were xamined histolbgically.

Clinical toxicity was minimal. Histological indings were confined to cytoplasmic alterations of liver cells.

New low dietary levels of Dieldrin (2.5 pm), lindane (50 ppm), and Toxaphene 50 ppm) were found occasionally effective n producing liver-cell changes. The previously reported minimal level for Chlordane 2.5 ppm) was confirmed.

The improvement in the sensitivity of the est is ascribed, at least in part, to the use f characteristic fatty inclusion bodies, lipospheres, as a measure of subtle cytoplasmic lteration.

Incidental pathological findings suggest he possibility of alterations in the permeaility of sinusoidal endothelium and the liver ell itself.

c/o Mrs. Maria Borisoff, 454 Vine St., Glendale , Calif.

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